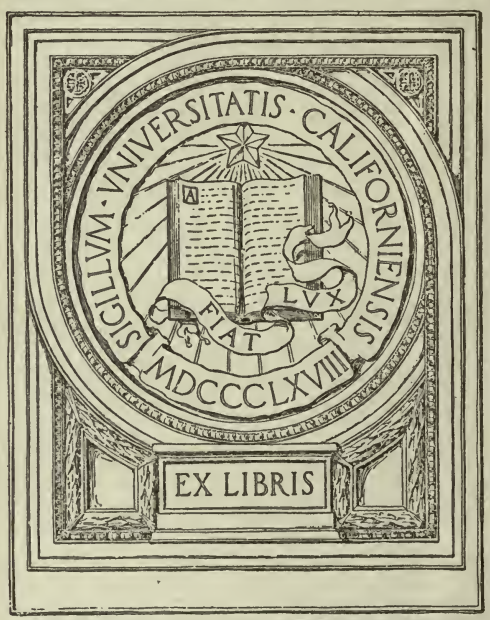

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GENERAL CHEMISTRY

OF THE

ENZYMES

BY

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TRANSLATED FROM
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PREFACE TO THE GERMAN EDITION

As the title of this book indicates, the author has attempted to review the more important facts of enzymology from a general standpoint and to fit them, so far as is possible, into their proper places in the fabric of general and physical chemistry. The aim has not been to give a complete synopsis of our knowledge of the enzymes, for already several such summaries are available.

It may perhaps be asked: Is the time yet ripe for giving a representation of the physical chemistry of the enzymes? The author feels that this question must be answered in the affirmative, although it is evident that extensive regions and important problems in the subject are still entirely untouched. The period during which the marshalling of facts was the most essential task was followed by one in which it was sought to harmonize the somewhat crude and imperfect experimental data with the laws of theoretical chemistry. The deviations from theory seemed to be wide and the peculiarities of enzymic reactions numerous. Only in the most recent times has the need for experimental revision of the quantitative data made itself felt. Improvements have been effected in the practical methods, while the factors participating in the reactions have become more clearly understood and are hence more fully taken into account. It is now being found that the results obtained from these more exact and comprehensive investigations correspond more closely with those required to satisfy physico-chemical theories. At the stage which has thus been reached in the development of enzymology a review such as that now published does seem to be justified. The author has therefore decided to allow the two reports on this subject which appeared in the " Ergebnisse der Physiologie " in 1907 and 1910, to be

arranged and issued in book-form, despite the fact that many problems still call for fuller treatment.

Although this monograph is intended more especially as an aid to scientific research in enzymology, yet the author trusts that it will be found useful by those concerned with the practical applications of enzymic actions. Thus an understanding of the dynamics of enzyme reactions is indispensable for the rational estimation of enzymic activities, such as that of pepsin in the gastric juice or that of diastases in malt, and these examples serve to show how theory may be of value to the physician and to the technical worker.

An appendix to the book contains a short sketch of experimental methods, more especially of those for which the original literature is not readily accessible. Professor Bertrand has kindly permitted the insertion of the tables prepared by him for use with his admirable method of estimating reducing sugars.

A considerable part of the labour involved in preparing this monograph has been undertaken by Miss Beth af Ugglas, Assistant in the Biochemical section of the Chemical Laboratory here and to her I wish to express my sincere thanks.

H. Euler.

Stockholm, January, 1910.

PREFACE TO THE ENGLISH EDITION

Although only two years have elapsed since the first publication of this work in the German language, the great energy with which the study of enzyme chemistry is being prosecuted has rendered necessary numerous additions and alterations. In view of the results of recent investigations, some of the sections, e.g., those concerned with the glucosides and the fermentation enzymes, have indeed been entirely rewritten.

To Mr. Pope's request to allow of the issue of an English edition of the book the author acceded the more readily because of the great success which has for a long time past attended enzymological research in English-speaking countries. At the present time, when various different paths have become clearly marked in general enzymic chemistry, the opportunity is welcomed of laying the author's views before English workers in this field.

To Mr. Pope the author is indebted, not only for a careful translation of his book, but also for certain improvements and additions in the part dealing with practical methods and for the references to the literature.

In the initial treatment of so extensive a subject as enzyme chemistry omissions are scarcely avoidable, and the author would be grateful to any readers who may contribute, either by sending him copies of their papers or by any other means, to render a subsequent edition more complete.

H. Euler.

Stockholm, March, 1912.

JOURNALS REFERRED TO BRIEFLY

Biochem. Z.: Biochemische Zeitschrift (Berlin).

Chem. Ber.: Berichte der deutschen chemischen Gesellschaft.

C. R.: Comptes rendus de l'Académie des Sciences (Paris).

H.: Hoppe-Seyler's Zeitschrift für physiologische Chemie.

Hofm. Beitr.: Hofmeister's Beiträge zur chemischen Physiologie und Pathologie.

Lieb. Ann.: Justus Liebig's Annalen der Chemie.

Pflüg. Arch.: Pflüger's Archiv für die gesammte Physiologie.

Soc. Biol.: Comptes rendus de la Société Biologique (Paris).

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GENERAL CHEMISTRY OF THE ENZYMES

INTRODUCTION

The name enzymes or unorganised ferments is given to animal or vegetable substances of unknown composition and constitution which, in the organism itself or even independently of the organ or cells in which they arise, are able to accelerate chemical reactions. The term enzyme is thus included in the much more general term, catalyst.

By catalyst we understand a substance which, without being required by the accelerated reaction or appearing among the final products, alters the velocity with which a chemical system strives to attain its final condition. But enzymes are—at least with the degree of purity in which they have as yet been obtained —rarely ideal catalysts. Only with difficulty, however, can a limit be set between these substances and ideal catalysts, this being greatly dependent on the experimental conditions.

The literature of the last few years shows, indeed, that a certain limitation in the meaning of the term enzyme is desirable; and, as a rule, those substances which are required in stoichiometric proportions by the reactions in which they participate are not regarded as enzymes.

With non-enzymic catalyses the quantity of the accelerating substance is mostly small compared with that of the substance acted on—indeed, an ideal catalyst should accelerate the transformation of unlimited amounts of "substrate." Even in the most minute quantities some enzymes certainly exert very considerable amounts of action; but usually their activity becomes limited with lapse of time and does not produce more than a certain amount of change. As we shall see later, this limitation is

due, partly to the participation of the enzyme in the equilibrium of the reaction and partly to the chemical instability of these substances. One property which the enzymes exhibit and which is generally regarded as characteristic of them, is that of becoming inactive if their solutions are heated for a longer or shorter time at a high temperature—about 100°. This is not an absolute criterion, as inorganic catalysts and enzymes do not exhibit any fundamental difference in this respect. As soon as any constituent of an organ which accelerates a reaction is explained chemically or identified with a known compound, no reason remains for terming it an "enzyme"; whether the term enzyme is to be retained for such substances, or whether the name—scientifically more accurate—of catalyst is to be employed, is entirely a matter for the future. But the choice of a definition is of subordinate importance, as we are, in many cases, so far removed from any chemical explanation of the enzymes that this term will certainly persist for a long time.

The distinction between the enzymes and the toxines is also, to some extent, arbitrary. Common to both classes of bodies are their origin in the living organism, their capacity of forming anti-bodies, and certain other properties, as also are their modes of action. On the other hand, the toxines are characterised with moderate sharpness by their poisonous action. We can, indeed, omit a treatment of this extensive subject all the more readily, as the physical chemistry of the toxines has undergone considerable development during recent years.

Of far greater importance than deciding how the enzymes are to be limited is the definition of the physical and chemical properties of typical representatives of these remarkable substances.

The aim in view is, of course, the exact description of the enzyme by a chemical formula and by constants characteristic of the pure substance. For the general chemistry of the enzymes, clear views concerning the degree of purity and the composition of the various members are of the greatest importance. In the first place, the enzymes must be prepared and analysed, and here physico-chemical investigations also afford valuable aid.

The first question to be decided is: Do the enzymes occur in a state of true solution, or must they be classed with colloidal substances? Or, speaking more strictly, which enzymes approach the one and which the other limiting case, and what can be

affirmed concerning their molecular magnitude and degree of dispersion? As criteria on these points serve diffusion, adsorption phenomena and also behaviour in the electric field.

More recent measurements have shown that the influence of temperature on enzyme action can be defined more exactly than earlier data would have led one to suppose, and the "temperature of destruction" and "optimum temperature," which give little information, are now replaced by well-defined physico-chemical magnitudes.

Undoubted and considerable success has followed the study, during the past few years, of activators or co-enzymes; mention need only be made here of the work of Harden and Young and of Buchner and Meisenheimer, which has led to the discovery of essential factors influencing alcoholic fermentation. Also, with reference to inactivators remarkable regularities have been observed, those concerning the influence of configuration calling for special mention. Further, the action of poisons is now so far understood that, in enzyme investigations, we can make use of substances which prevent bacterial infection and yet have no harmful effect on the enzyme, thus avoiding the errors which have been so often caused in work of this kind by insufficient disinfection.

Although the majority of the anti-bodies, so important physiologically, are classed among the toxines, yet such a large number of observations have been made on the anti-ferments that these must not remain unnoticed.

When we have, in the first part of this book, obtained information concerning the chemical facts of enzymology, we must turn to the second part of the question: In what manner is a reaction induced or accelerated by an enzyme and how do enzymic reactions proceed?

In the first place, we will consider the laws of chemical dynamics which come into play in enzymic reactions and, in particular, the results which have been obtained from a study of catalysts. Comparison of non-enzymic reactions with enzymic changes will show us that the same processes are being dealt with in the two cases and that the deviations from the classical exampies of chemical dynamics, exhibited by many enzyme reactions, are readily explained by the simple assumption that enzyme and

substrate unite to form more or less stable complexes, which are to be regarded as the " active " molecules and hence bring about the reaction. This assumption is adhered to all the more strongly, because it corresponds with our general conception of the rôle of catalysts.

In the fourteen years which have passed since the synthesis of isomaltose by maltase was discovered (Croft Hill), the number of enzymic syntheses has become quite considerable. Not only do we now know enzyme reactions which proceed in both directions, but in several cases the synthetic action of the enzyme has been separated, and caused to take place apart, from the decomposing action. A knowledge of these syntheses is naturally of the utmost importance for the biochemistry of animals and plants.

For chemistry in general these processes are the more important, since, as is well known, the enzymes are extremely sensitive towards the steric configuration of the substrate and lead to the formation of asymmetric products. The enzymes hence place us in a position to effect asymmetric syntheses.

CHAPTER I

SPECIAL CHEMISTRY OF THE ENZYMES

NOMENCLATURE

The already large and rapidly increasing number of enzyme actions necessitates a rational system of nomenclature. According to a proposal made by Duclaux (see Bourquelot, Les ferments solubles, Paris, 1896), the name of the enzyme is derived from that of the substance on which it acts: for example, lactase is the enzyme which decomposes lactose. Unfortunately, many departures have been made from this principle. In cases where the name derived from that of the substrate is not sufficiently definite, E. O. von Lippmann (Chem. Ber., 1903, **36**, 331; see also Buchner and Meisenheimer, Chem. Ber., 1905, **38**, 621) proposes that both the name of the substance acted upon and that of the (principal) product formed from it should be indicated in the name of the enzyme; for example, amylo-maltase would be the enzyme which forms maltose from starch. But as this enzyme is not a maltase, but belongs rather to the class of amylases, it would be more convenient to term it malto-amylase. Names in general use, such as pepsin, zymase and erepsin, are retained. Although the employment of a rational method of naming enzymes is to be desired, yet, on the one hand, the right to give or alter a name must be left with the discoverer, and, on the other, the region of action and the individuality of enzymes, like pepsin are not yet so completely determined as to allow of the adoption of a perfectly suitable name.

According to a suggestion by the author (H., 1911, **74**, 13), the names of synthesising enzymes should be made to indicate the substances which they form and to terminate with the syllable "ese"; thus phosphatese would be the enzyme which synthesises organic esters of phosphoric acid, and nitrilese (δ-nitrilase according to Rosenthaler) that which forms nitriles.

CLASSIFICATION

Since very little is known concerning the nature of the enzymes, the classification of these bodies is based on the chemical reactions which they induce. And it is to be expected that the classification indicated by the chemical actions would also be brought out in the physical and chemical properties of the enzymes.

An enumeration of all the enzymes described in the literature of the subject does not fall within the scope of this work; indeed, there are a very large number of such substances, the individuality of which has not been sufficiently well established.

The following summary serves rather to indicate, in a general way, typical reactions in which enzymes play a part.

Reaction.	Substrate.	Products.	Enzyme.
Hydrolyses	**Esters:**	Fatty acids +alcohols	Esterases:
	Fats	Higher fatty acids +glycerol	Lipases
	Lower esters	Lower fatty acids +alcohols	Butyrases
	Chlorophyll +alcohol	Crystalline chlorophyll +phytol	Chlorophyllase
	Higher carbohydrates:		
	Cellulose	.....	Cellulase
	Hemicellulose	.....	Cytase
	Starch, glycogen	Maltose (dextrins)	Amylases and amylo-pectinases
	Inulin	Fructose	Inulinase
	Pectoses	Pectin	Pectase
	Glucosides including Polysaccharides:	Hexoses and glucoside-residues	
	α-Glucosides	Glucose + sugar, alcohol or phenol-residue	α-Glucosidase (Maltase)
	β-Glucosides	Glucose + sugar, alcohol or phenol-residue	β-Glucosidase (Emulsin)
	β-Galactosides	Galactose + sugar, alcohol or phenol-residue	Lactase
	Fructosides	Fructose +sugar residues	Invertase
	Other glucosides	Other sugars +phenols, etc.	Rhamnase, Myrosin, etc.
	Phytin	Inositol +phosphoric acid	Phytase
	Hexosephosphate	Hexose +phosphate	Hexosephosphatase
	Digallic acid (Tannin)	Gallic acid	Tannase
	Carbamide derivatives:		
	$R \cdot CO \cdot NH \cdot R'$	$R \cdot COOH + R' \cdot NH_2$	Carbamases, proteinases
	Proteins	Albumoses, peptones	Pepsin, papain
	Proteins, albumoses, peptones, peptides	Peptides, amino-acids	Trypsin, erepsin
	Arginine	Urea +ornithine	Arginase
	Nucleic acids	Nuclein bases +phosphoric acid	Nuclease

Reaction.	Substrate.	Products.	Enzyme.
Hydrolyses	**Acid amides:**		
	Urea	Carbon dioxide $+NH_3$	Urease
	Amines:		Desamidases
	Amino-acids	Hydroxy-acids $+NH_3$	Desamidase
	Guanine	Xanthine $+NH_3$	Guanase
	Adenine	Hypoxanthine $+NH_3$	Adenase
Decompositions	Hydrogen peroxide	Molecular oxygen $+H_2O$	Catalases
Syntheses	Hydroxynitriles	Aldehyde $+HCN$	Nitrilases
	Benzaldehyde $+HCN$	Mandelic acid nitrile	*d*-Oxynitrilese
	Na_2HPO_4 +carbohydrate	Ester of carbohydrate $-$phosphoric acid	Phosphatese
Transference of oxygen	Peroxides	Reduction products of peroxides $+O$	Peroxydases
Unknown processes accompanied by coagulation	Casein	Paracasein (+whey-albumen)	Chymosin
	Fibrinogen	Insoluble fibrin	Fibrin ferment (thrombin)
	Pectins	Pectinates	Pectinase
Fermentations	Glucose	Lactic acid	Zymase of lactic acid bacteria
	Glucose, fructose, mannose, galactose	Alcohol $+CO_2$	Zymase (sum-total of the enzymes of alcoholic fermentation)
Oxidations	Phenols	Quinones	Phenolases
	Aldehydes	Acids	Aldehydases
	Alcohol	Acetic acid	Alcoholoxydase of acetic acid bacteria

Certain enzymes which exert actions other than those given above are mentioned in the Appendix to Chapter I.

SPHERE OF ACTION OF THE ENZYMES. THEIR PREPARATION AND PURIFICATION

In this section are given such data as appear necessary for understanding the general behaviour of the enzymes.

The dynamics of the enzymes suffers in considerable measure from the disadvantage that we know nothing of the composition of these bodies and hence can form beforehand no idea of the chemical processes taking place during their action. It is, there-

fore, all the more necessary to investigate experimentally all the factors influencing enzymic reactions, in order to avoid the danger of missing a secure foundation for the theoretical treatment of the subject.

Especially would the author point out that it is not possible to pay too much attention to the preparation and purification of enzymes for use in physico-chemical measurements.

Enzyme preparations are obtained either by subjecting the organs to pressure or by extracting them with suitable solvents. The consistency of the starting material, the admixtures which are always present, the age and especially the previous history of the preparation, influence not only the intensity, but also the mode of action of the enzyme to a greater extent than many investigators have supposed. A knowledge of the material is hence indispensable to a critical examination of the experimental results.

Extracts or preparations of organs often exert several enzymic actions at the same time; thus, to choose an example from recent literature, a preparation from croton seeds has been found by Scurti and Parrozzani (Gazzetta Chim. Ital., 1907, **37**, i, 476) to hydrolyse, not only fats and esters of monobasic acids, but also cane sugar and proteins. From these results, it should be concluded, not that an enzyme exists possessing a general hydrolytic capacity, but that the preparation employed contains several enzymes.

In order to study the separate components, the different actions have to be separated, and when a preparation has been obtained which exhibits only a single reaction, it is termed biologically pure. There still remains, however, the possibility that the various stages of the reaction are accelerated by different constituents of the enzyme. It has, for example, been found to be probable that, in the hydrolysis of amygdalin, three enzymes take part, one of them effecting the resolution into glucose and the glucoside of mandelic acid nitrile, a second hydrolysing the latter compound to mandelonitrile and glucose, while the third decomposes the nitrile into benzaldehyde and hydrocyanic acid.

Even those enzymes which are biologically the purest are still very far removed from the state of chemical purity and we possess—and on this stress must be laid—no certain knowledge

that even an approximate isolation of any hydrolytic enzyme has yet been attained. This is explained by the instability of the enzymes, which, when subjected to protracted and energetic purifying processes, become inactive, so that their presence can no longer be detected; and also by the extremely small concentrations in which the enzymes always seem to occur in nature, and by the large amounts of impurities—especially of colloidal substances—contained in the extracts.

Of the oxydases, which are to some extent stable to heat, we have chemical knowledge of at least one member.

We shall see later that the behaviour of enzymes towards external influences, such as acids, alkalies, co-enzymes, etc., is often determined, wholly or partially, by the impurities present.

On account of the importance which processes of purification have for enzymology, the methods employed are treated somewhat in detail.

As may be again mentioned, consideration of the whole of the literature on the different enzymes does not come within the limits of this work. In the first place, investigations will, of course, be omitted to which lasting value cannot be ascribed, and no attention will be paid to those dealing with purely physiological questions and with the distribution of the enzymes in the animal and vegetable kingdoms, since these are not directly connected with the general chemistry of the enzymes.

ESTERASES

The usual action of these enzymes consists in the hydrolysis of esters. The enzymes of this group prove to be more or less markedly specific, as will be shown more in detail in Chapter VIII. It must, however, be mentioned that the lipase of the stomach decomposes, not only true fats, but also the lipoids, lecithin, jecorin and protagon (P. Mayer, Biochem. Z., 1906, **1**, 81; Schumoff-Simanowski and Sieber, H., 1906, **49**, 50). Also pancreatic juice, according to Abderhalden and others, decomposes lecithin [but a negative result was obtained by Kalaboukoff and Terroine (Soc. Biol., 1909, **66**, 176)]. A special class is formed by the

Lipases, which resolve more especially the natural fats, i.e., the glycerol esters of palmitic, stearic and oleic acids.

Animal lipases play an important part in the stomach (gastric juice and mucous membrane [1]), pancreas [2] and intestines [3] of the higher animals. Also serum contains lipases (Neuberg and collaborators) and, according to Pagenstecher (Biochem. Z., 1909, **18**, 285), this is the case with all the organs, especially the liver and spleen, of the ox. These animal enzymes decompose both animal and vegetable fats and oils.

A lipase has also been found in the albumen of hens' eggs. In general, it is relatively difficult to obtain active extracts from animal organs containing lipase and Connstein is of the opinion that it is best to employ the pancreatic juice of the crushed glands themselves. Aristides Kanitz, however, seems to have prepared active glycerol-extracts (H., 1905, **46**, 482), and Lewkowitsch and Macleod have worked with aqueous lipase solutions which attack neutral fat (Proc. Roy. Soc., 1903, **72**, 31). The extraction of lipase-preparations from the pancreas has been investigated in detail by Dietz and Pottevin (Bull. Soc. Chim., 1906, [iii], **35**, 693; see also E. Baur, Zeitschr. f. angew. Chem., 1909, **22**, 97). O. Rosenheim (Journ. of Physiol., 1910, **40**) has recently made the interesting observation that the lypolytic enzyme can be separated from its activator or co-enzyme by mere filtration of the glycerol extract of pancreatic lipase. The substance remaining on the filter is sensitive to heat, whilst that in the filtrate is stable to heat. A mixture of these two exerts enzymic action, but each separately is inactive.

There is a great amount of contradiction among the results obtained with the lipases; it does, however, seem established that the lipases of the stomach and pancreas do not exhibit identical properties. The two animal lipases appear to be related in the same manner as the corresponding proteolytic enzymes, pepsin and trypsin; but they both differ essentially from the lipases of seeds.

[1] F. Volhard, Zeitschr. klin. Med., 1901, **42**, 414 and **43**, 397; W. Stade, Hofm. Beitr., 1903, **3**, 291; A. Zinsser, Hofm. Beitr., 1906, **7**, 31; A. Fromme, Hofm. Beitr., 1905, **7**, 51; A. Falloise, Arch. Internat. de Physiol., 1906, **3**, 396, and 1907, **4**, 405.

[2] H. Engel, Hofm. Beitr., 1905, **7**, 77; Umber and Brugsch, Arch. f. exp. Path., 1906, **55**, 164.

[3] W. Boldyreff, H., 1907, **50**, 394.

Vegetable lipases are obtained from oily seeds, especially of Ricinus. The first work on these lipases was carried out by Reynolds Green (Proc. Roy. Soc., 1890, **48**, 370) and Sigmund. Further contributions to the knowledge of them are due to Connstein, Hoyer and Wartenberg (Chem. Ber., 1902, **35**, 2988), Nicloux (Soc. Biol., 1904, **56**, 840 and Proc. Roy. Soc., *B.*, 1906, **77**, 454), H. E. Armstrong (Proc. Roy. Soc., *B.*, 1905, **76**, 606) and others. A very good monograph has been written by Connstein for the "Ergebnisse der Physiologie" (1904, **3**). E. Rouge (Centralbl. f. Bakt., 1907, II, **18**, 403) gives a résumé of the literature dealing more particularly with vegetable lipases.

Ricinus-lipase is only active in relatively strongly acid solution, and in the seeds, it is activated by the lactic acid present (Hoyer, H., 1906, **50**, 414). According to Braun and Behrend (Chem. Ber., 1903, **36**, 1142, 1900), the seeds of Abrus precatorius, which are nearly related to Ricinus seeds, decompose fats in neutral solution, but the effect is comparatively slight. Characteristic of the Ricinus-enzyme, as of most true lipases, is its insolubility in water; it is hence necessary to bring the pressed mass, remaining after the removal of the oil from the seeds, into intimate contact with the fat-emulsion.

As to the other sources of plant lipases, mention may be made of the fungi, both higher and also lower, like Penicillium (Gérard, C. R., 1897, **124**, 370; Camus, Soc. Biol., 1897, **49**, 192), Aspergillus niger (Camus, loc. cit.) and especially yeast (Delbrück). Lipases have also been detected in numerous bacteria; they cause the rancidity of butter and other natural fats (for the literature see Fuhrmann, Bakterienenzyme, Jena, 1907).

Butyrases. Against the extended application of the esters of the lower fatty acids and the monoglycerides to the study of the lipases, objection has often been raised. In particular, Arthus (Soc. Biol., 1902, **53**, 381) and also Doyon and Morel (C. R., 1902, **134**, 1001 and 1254) have pointed out that Hanriot's experiments with monobutyrin (Soc. Biol., 1896, **48**, 925; C.R., 1896, **123**, 753) which challenge criticism in many directions, give no information as to the presence and action of

the true lipases. A distinction must therefore be drawn between the lipases and esterases (butyrases). The latter enzymes, which occur abundantly in many juices and organs (blood-serum, liver, kidneys) decompose not only the monovalent alkyl and the glyceryl esters of the lower fatty acids, but also amyl salicylate (H. Chanoz and H. Doyon, Soc. Biol., 1900, **52**, 116, 717) and similar compounds. Schmiedeberg's histozyme is perhaps identical with these enzymes.

Dakin has effected asymmetric ester-decompositions by means of liver-esterases; these will be considered in Chapter VIII. Detailed studies on the same enzymes are due to Kastle, Loevenhart and Elvove, whose quantitative measurements will be referred to in the third chapter. They give the following method for the

Preparation of liver-esterases. The macerated liver (10 grms.) is extracted with water (100 c.c.) and the extract filtered through a linen cloth. Twenty c.c. of this extract are diluted with 72 c.c. of water and 8 c.c. of a 0·01N-solution of hydrochloric acid added. When this mixture is heated to 40°, a heavy precipitate of protein separates and, on filtering, a clear golden-yellow solution is obtained.

The action of pancreas-lipase is participated in by a co-enzyme (R. Magnus, H., 1904, **42**, 149), which is stable at a boiling temperature and the essential constituents of which are alkali salts of the bile acids (Magnus, H., 1906, **48**, 376; see also Chapter V).

Among the esterases must also be classed

Chlorophyllase. This very interesting enzyme, which was discovered and described by Willstätter and Stoll (Lieb. Ann., 1911, **378**, 18), accompanies chlorophyll and is wide-spread in its occurrence. The reaction which it produces is an alcoholysis.

Chlorophyll contains three carboxyl groups, one of which is apparently free, and the others esterified with a methyl and a phytyl (from phytol) group respectively. Only the latter reacts with the alcohol, and then only under the influence of the enzyme, the phytoxyl group being replaced by ethoxyl.

"In its action, chlorophyllase cannot be replaced by other esterases. On the other hand, for the enzyme found in the leaves, chlorophyll is a specific substrate. With phæophytin

this enzyme does not react so well and with an ordinary wax no reaction takes place."

ENZYMES OF THE HIGHER CARBOHYDRATES

Cytases. Whether true cellulose is decomposed enzymically is still uncertain. More is known, especially from the investigations of Mac Gillawry, of H. T. Brown and Morris (Journ. Chem. Soc., 1890, **57**, 497), of Reynolds Green (Annals of Bot., 1893, **7**, 93) and, recently, of Schellenberg (Flora, 1908, **98**, 257), of the action of cytase, the substrates of which are the hemicelluloses and their reaction-products, mannose and galactose; also pentose-polysaccharides —the pentosans—are decomposed, but it is not yet known whether the hydrolysis is complete. Such enzymes occur in the intestines of herbivorous animals, in wood-destroying fungi (Czapek, Lotos, 1898, **46**, 235; Schorstein, Centrabl. f. Bakt., 1902, II, **9**, 446) and in bacteria. Also hydrocelluloses, which, so far as is known, are nearly allied to the hemicelluloses, are decomposed by cytases. To the same group of enzymes belong caroubinase, which dissolves the caroubin in the carob (Ceratonia siliqua) (Effront, C. R., 1897, **125**, 116) and the enzyme described by Bourquelot and Hérissey (C. R., 1899, **129**, 228, 391, 614; 1900, **130**, 42, 340, 741) as seminase, which occurs in lucerne, Trigonella and other plants. As well as in plants, cellulases or cytases are found in the animal body, especially in the intestines of graminivorous animals (H. T. Brown, Journ, Chem. Soc., 1892, **61**, 352), in snails (Biedermann and Moritz, Pflüg. Arch., 1898, **73**, 236) and in fishes (Knauthe). Experiments by the author (Zeitschr. f. angew. Chem., 1912, **25**, 46) indicate the occurrence in Merulius lacrimans (dry-rot fungus) of an enzyme which decomposes cellulose-dextrin.

Amylases. These enzymes—more accurately termed malto-amylases—include all those which break down starch and glycogen forming maltose. Very little that is definite can be asserted with regard to the individuality of the amylases.

After the view had been expressed by Brown and Morris and by Reynolds Green that at least two enzymes

showing different biological relations are to be distinguished (diastase of translocation and diastase of secretion), Maquenne (C. R., 1906, **142**, 124, 1059, 1387) carried out a series of notable investigations which explained the saccharification from a chemical point of view. Starch consists, indeed, of 80–85% of amylose and 15–20% of amylopectin. Amylase attacks dissolved amylose and also "soluble starch" very readily, but acts on amylopectin (starch-paste) very slowly. Amylopectinase, on the contrary, saccharifies amylopectin (starch-paste) with great ease. The diastases of the very varying organs of plants and animals must contain both of these diastatic enzymes. That the saccharifying and liquefying actions of the "diastases" are often parallel has been shown more especially by an investigation made by Chrzascz (Zeitschr. f. Spiritusind., 1908, **31**, 52). Another recent noteworthy contribution on vegetable diastatic enzymes is due to Butkewitsch (Biochem. Z., 1908, **10**, 314). Further investigations in this direction are, however, desirable, as well as a more detailed study of the diastatic decomposition of glycogen.

Also the individuality of the true amylases, apart from amylopectin, is doubtful, if we consider the far-reaching decomposition necessary in order to pass from the highly-condensed starch through the dextrins to maltose. After Miss Tebb (Journ. of Physiol., 1894, **15**, 421), Brown and Morris, Röhmann (Chem. Ber., 1894, **27**, 3251), Hamburger (Pflüg. Arch., 1895, **60**, 543) and Beijerinck (Centralbl. f. Bakt., 1895, II, **1**, 221) had detected, in the mixture of enzymes which saccharifies starch, an enzyme which effects the transformation starch-to-maltose and another which further breaks down the maltose to glucose, Wijsman (Rec. Trav. Chim. Pays-Bas, 1890, **9**, 1), Pottevin and others assumed that the reactions starch-to-dextrin and dextrin-to-maltose are also effected by separate enzymes,[1] and recently Ascoli and Bonfanti (H., 1904, **43**, 156) speak of several amylases.

Occurrence. Enzymes which attack glycogen and starch are, as was discovered by Claude Bernard, widespread in the animal

[1] Lintner's statement that isomaltose is formed during the diastatic conversion of starch must, after subsequent work, more especially by Ling and Baker (Journ. Chem. Soc., 1895, **67**, 702) and by Brown and Morris (ibid., 709), be regarded as disproved.

kingdom. Their occurrence in blood-serum which was detected by this investigator has been examined more closely by Bial, Pick, and Ascoli and Bonfanti (H., 1904, **43**, 156). According to Nasse such an enzyme occurs in muscle-plasma, and this was confirmed by Halliburton (Journ. of Physiol., 1887, **8**, 182). Carlson and Luckhardt (Amer. Journ. of Physiol., 1908, **23**, 148) have found amylase in numerous other body-liquids. The fact that saliva dissolves starch has been known much longer, and this property was ascribed by Leuchs in 1831 to an enzyme, ptyalin. Foster and von Wittich also found amylolytic enzymes in a great number of organs. In addition to the liver and pancreas, the muscles are especially rich in an enzyme which attacks glycogen; and Mendel and Saiki (Amer. Journ. of Physiol., 1908, **21**, 64), by experiments on the pig, found that this is the case in the embryo state, while other organs of different animals usually become richer in enzyme as development proceeds (Pugliese and others). According to Roger (Soc. Biol., 1908, **64**, 1137), an amylase occurs also in hens' eggs (white and yolk) and is partially soluble in ether.

Of no less biological importance than animal amylases are those of plants. The starch-decomposing action of germinated barley was discovered by Kirchoff as long ago as 1814. An enzyme-preparation was made in 1833 by Payen and Persoz and was named "diastase"; this term is also much used at the present time, but, in the interests of as rational as possible a nomenclature, it should be replaced by the terms amylase, amylopectinase, etc.[1] The whole of the saccharifying enzyme-preparation together, that is, the mixture of amylase, dextrinase, etc., may meanwhile be called diastase.[2]

In accordance with the function of the diastases of effecting the metabolism of the polysaccharides, these enzymes are widespread in all parts of plants, and are especially abundant in shoots and leaves, particularly with the Leguminosæ and grasses (Brown and Morris,

[1] Wijsman's nomenclature is by no means an acceptable one. If two enzymes really take part in the formation of maltose, they should be distinguished as amylase and dextrinase.

[2] It is most desirable that the use of the term "diastases" as a generic name for enzymes should be abolished from the French literature. For this use of the term there is, indeed, a historical explanation, but there is no justification for its continuance, especially as it often gives rise to misunderstanding.

Journ. Chem. Soc., 1893, **63**, 604). Amylases have further been detected in potatoes and the sugar-beet; also in germinating, starch-containing pollen-grains (Reynolds Green), in the bark of many plants (Butkewitsch, Biochem. Z., 1908, **10**, 314), in the sap, in many higher and lower fungi, especially in several species of yeast—here the diastase may be related to the glycogen-content—and finally in bacteria.

Special mention must be made of the so-called taka-diastase, the mixture of saccharifying enzymes from Aspergillus oryzae, a fungus contained in koji-yeast. It saccharifies starch and indeed, according to Stone and Wright, and Takamine, more energetically than does malt-diastase. Experiments with the view of preparing it in a pure state were made by Wroblewski (Chem. Ber., 1898, **31**, 1130).

Preparation. Of the animal amylases, the ptyalin of saliva is the best suited for preparation. According to J. Cohnheim (Virch. Arch., 1865, **28**, 241) the saliva is precipitated with freshly-prepared calcium phosphate. From the precipitate the ptyalin is dissolved by means of water, and the aqueous solution precipitated with alcohol. Another method is given by Krawkow (J. Russ. Phys. Chem. Soc., 1887, **19**, 387), who precipitates saliva-diastase by ammonium sulphate. Direct precipitation of the saliva with alcohol also yields a saccharifying preparation.

Cohnheim has found saliva-diastase to be free from protein, but he does not state on what absolute quantities of the preparation the tests were made.

Von Wittich takes up pancreas-diastase in anhydrous glycerol.

Larger and purer yields of amylase are obtained from vegetable material. From malt Lintner prepared diastase as follows: one part of green malt (or air-dried malt) was extracted for 24 hours with 2–4 parts of 20% alcohol, the extract being precipitated with 2–5 times its volume of absolute alcohol and the precipitate washed with absolute alcohol and ether.

Loew (Pflüg. Arch., 1882, **27**, 203; 1885, **36**, 170) steeps germinated barley in a little water and then extracts with 4% alcohol. He precipitates the extract with lead acetate, suspends the precipitate in water, removes the lead from the solution by means of hydrogen sulphide, and finally precipitates the diastase with a mixture of alcohol and ether.

Osborne and Campbell (Journ. Amer. Chem. Soc., 1896, **18**, 536) and also Wroblewski (H., 1897, **24**, 73) salt out the diastase with ammonium sulphate.

Effront (Enzymes and their Applications, London and New York, 1902, pp. 104 et seq.) proposes the extraction of malt with water and, in order to diminish the quantity of the extractive material possessing no diastatic action, he induces alcoholic fermentation in the infusion by yeast previously rendered very poor in nitrogen. Effront states that the fermentation destroys a large quantity of carbohydrates, removes considerable quantities of proteins and salts, and leaves the diastase absolutely untouched.

Wroblewski (Chem. Ber., 1897, **30**, 2289) gives the following method:

Finely-ground malt is extracted, first with 70%, and then twice with 45% alcohol. Sufficient strong alcohol is added to the last two extracts to bring the alcohol-content to 70%. The precipitate formed is washed with absolute alcohol and ether and dried in a vacuum.

Like Osborne and Campbell (loc. cit.), Wroblewski (Chem. Ber., 1898, **31**, 1130) effected further purification by salting out with ammonium sulphate.

Wroblewski considered that, as a result of these experiments, he had shown with certainty that diastase is a protein substance nearly allied to the albumoses, whilst, according to T. B. Osborne (Chem. Ber., 1898, **31**, 254), diastase is a protein-like substance or "a compound of an albumin with a proteose." For his most active preparation he gives the following composition (calculated for ash-free substance): C, 52·5; H, 6·72; S, 1·90; N, 16·10%. The solution gives the characteristic reactions of the proteins. Wroblewski's purest preparation had a nitrogen-content of 16·5%.

If, however, the recent researches of S. Fränkel and Hamburg (Hofm. Beitr., 1906, **8**, 389) should be confirmed, diastase contains neither protein-groups nor reducing sugars. The non-enzymic substances were precipitated with lead acetate, the solution sterilised by filtration and further purified by fermentation with yeast rendered poor in nitrogen and subsequent filtration through a Pukall filter. After drying in a vacuum, the syrupy liquid yields a powder free from fermentable and reducing

sugars and from protein. It represents a very active substance which does not give the biuret reaction or reduce Fehling's solution but shows a faint Millon's reaction; it also gives Molisch's reaction and the pentose reaction slightly. When dialysed into spring-water, the dissolved diastases are separated into two principal groups: the saccharifying diastases diffuse through the membrane, whilst the liquefying ones remain.

A distinct advance seems to have been made by H. C. Sherman and M. D. Schlesinger (Journ. Amer. Chem. Soc., 1911, **33**, 1195). They found that pancreas-diastase keeps well in 50% alcohol, and they purified such a solution by dialysis. A very active preparation (saccharifying power, 5000 on Lintner's scale at 40°) contained 53·0% C, 6·6% H and 15·6% N.

Inulinase. In addition to hemicelluloses, starch and glycogen, another carbohydrate, inulin, also occurs as a reserve material. The enzyme, inulase or inulinase, accompanying this, decomposes inulin into its simplest component, fructose. Starch is not attacked by inulinase.

Reynolds Green (Annals of Bot., 1888, **1**, 223) discovered this enzyme, which occurs in many of the Compositæ, in the tubers of Helianthus tuberosus (artichoke). Bourquelot (Bull. Soc. Mycol., 1893, **9**, 230; 1894, **10**, 49) detected inulinase in Aspergillus niger and isolated it from the mycelium of this fungus. The enzyme appears to be widespread in the Eumycetes; Dean (Bot. Gaz., 1903, **35**, 24) found it also in Penicillium glaucum. The best medium for its action is 0·001% hydrochloric acid.

The enzymes which decompose trisaccharides, such as melicitase, etc., have not yet been sufficiently individualised.

THE ENZYMES OF THE GLUCOSIDES AND DISACCHARIDES

E. Fischer has stated that the disaccharides may be regarded as glucosides and can be classified with these according as they contain the glucose in the α- or β-form. From the results of E. F. Armstrong (Journ. Chem. Soc., 1903, **83**, 1305) and C. S. Hudson (Journ. Amer. Chem. Soc., 1909, **31**, 1242), it appears that α-glucosides, which were originally characterised

by the fact that they are hydrolysed by a constituent of yeast-extract, generally yield α-glucose.

β-Glucosides are hydrolysed by a component of almond extract, and from such a glucoside Hudson obtained β-glucose.

α-Glucosidase; maltase. As α-glucosidases will be designated those enzymes which hydrolyse α-glucosides specifically. They are therefore limited, on the one hand, by β-glucosidase, which acts only on β-glucosides, and, on the other, by lactase and invertase, which accelerate the hydrolysis of the β-galactosides or fructosides.

Occurrence. In both the animal and vegetable kingdoms, maltase almost always accompanies the diastases, from which it cannot often be separated. Thus, this enzyme has been found in blood and in serum (Gley and Bourquelot, Soc. Biol., 1895, **47**, 247; Hamburger, Pflüg. Arch., 1895, **60**, 543; Tebb, Journ. of Physiol., 1894, **15**, 421; Fischer and Niebel, Sitzungsber. K. Akad. Berlin, 1896, 73); also in many tissues (Shore and Tebb, Journ. of Physiol., 1892, **13**, 19), especially in the liver, intestines and pancreas. As Miss Tebb found, the maltase can be extracted from these organs—in both the fresh and dried states—by means of chloroform water; the opposite statement of Brown and Heron (Proc. Roy. Soc., 1880, **30**, 393) is thus contradicted.

The maltases occur in great abundance in the vegetable kingdom. Their occurrence in malt and in yeast must be especially mentioned. These two maltases do not appear to be absolutely identical (Fischer, H., 1894, **26**, 74). The lactic acid yeasts and also kephir-grains always contain lactases in place of maltases. Saccharomyces Marxianus contains no maltase, but only invertase (E. C. Hansen; E. Fischer and P. Lindner, Chem. Ber., 1895, **28**, 984). Excepting in this case, it can be said that invertase always accompanies maltases in yeast-extracts; according to Beijerinck and to E. Fischer and P. Lindner (Chem. Ber., 1895, **28**, 984), invertase is lacking in Saccharomyces octosporus. In Saccharomyces apiculatus neither maltase nor invertase is found.

Preparation. As starting material for the preparation of the yeast-enzymes, maltase and invertase, it is best to employ pure cultures. The yeast is used as fresh as possible and is well pressed and, according to E. Fischer (H., 1898, **26**, 74), ground and well shaken two or three times with the ten-fold quantity of water. Removal of the mother-liquor is effected

most suitably by a Pukall flask-filter. The yeast is pumped as dry as possible and is then spread out in as thin a layer as possible on porous tiles and dried in the air at the ordinary temperature. Under these conditions it gradually shrivels up and assumes a dark-grey colour. After 1–2 days, it is powdered as finely as possible and left to dry in the air until it forms a loose powder. The final drying may also be carried out at 30–35°. In this state the yeast can be kept for months without the maltase being destroyed. When the enzyme is to be used, the yeast is extracted with 10–15 times the quantity of water for 12–20 hours at 30–35°, with occasional shaking, the liquid being then filtered through paper. Toluene serves as a suitable antiseptic.

Röhmann (Chem. Ber., 1894, **27**, 3251) heated the yeast for an hour at 105–110° before extraction, but this procedure, according to Croft Hill, is not to be recommended. The latter investigator gives the following method (Journ. Chem. Soc., 1898, **73**, 636): Good, pressed bottom-yeast is washed three times with distilled water by decantation, collected on a covered filter, spread out on a porous support and dried in a vacuum over sulphuric acid. The yeast dries in about two days and is then powdered and sieved through a cloth, a yellowish-white powder being obtained. This powder is then spread out on a double layer of fine tulle over the mouth of a glass vessel in an oven previously heated to 40°. In successive quarters of an hour, the temperature is raised to 60°, 70°, 90° and 100°, the last being maintained for 15 minutes, after which the preparation is allowed to cool in a desiccator. The yeast is then weighed, ground in a mortar with 10 times its weight of 0·1% sodium hydroxide solution, filled into flasks with addition of toluene and left at the room-temperature for 3 days. The extract is now filtered, first through paper and afterwards through a Chamberland filter. If 1 c.c. of this fresh extract is added to 20 c.c. of 2% maltose solution at 30°, about 20% of the sugar is hydrolysed in 40 minutes.

Different yeasts appear to contain widely varying proportions of maltase, so that not every species of yeast is suitable for the preparation of maltase.

Trehalase. Trehalose, a disaccharide composed of two molecules of glucose, is hydrolysed by an enzyme which was

found by Bourquelot in Aspergillus and other fungi, by Fischer (H., 1898, **26**, 79) in the diastase of green malt and in yeasts of the Frohberg type, and by Kalanthar (H., 1898, **26**, 97) in various other yeasts. Whether the enzyme, which acts best with a very slight concentration of hydrogen-ions, really differs from maltase is not yet established; Bourquelot (Soc. Biol., 1895, **47**, 515) regards it as a separate enzyme, but Fischer is not in agreement with this opinion.

The β-glucosidases hydrolyse β-methylglucoside and also most of the natural glucosides, which are on this account placed in the β-series.[1]

It has been shown recently by Hudson and Paine (Journ. Amer. Chem. Soc., 1909, **31**, 1242) that the hydrolysis of a typical natural glucoside, salicin, under the influence of emulsin, yields β-glucose. It is better here not to apply the ordinary principles of nomenclature, but to name the glucosides according to the form of glucose to which they give rise and the enzymes so that they refer to the glucosides characterised in this way.

Of special glucosido-glucoses hydrolysed by emulsin, mention may be made of:

Isomaltose (E. Fischer, Chem. Ber., 1895, **28**, 3024; compare also E. F. Armstrong, Proc. Roy. Soc., *B*, 1905, **76**, 592).

Gentiobiose (Bourquelot and Hérissey, C.R., 1902, **135**, 399).

Cellose or Cellobiose (E. Fischer and G. Zemplén, Lieb. Ann., 1909, **365**, 1).

Cellase. From the results of fractional filtration of extracts of Aspergillus niger by Holderer's method, G. Bertrand and M. Holderer (C. R., 1909, **149**, 1385 and 1910, **150**, 230) assume the existence of an enzyme which differs from β-glucosidase and acts specifically on cellose. An enzyme extracted from apricot seeds hydrolyses only cellose and not trehalose.

[1] The opportunity must not be neglected of pointing out that E. Fischer, who introduced this method of reasoning, has issued a warning that it must not be regarded as absolutely safe (Lieb. Ann., 1909, **365**, 1): "For it might be assumed that one and the same enzyme hydrolyses both the alcohol-glucosides and the glucosido-glucoses. But as long as no pure enzyme is obtained, complicated mixtures like emulsin or yeast-extract having to be used, no proof of this exists."

Cellase occurs, together with other enzymes, in apricot kernels, almonds, barley, and the mycelium of Aspergillus.

β-Methylgalactosides are also hydrolysed by the enzymes of the almond (E. Fischer, Chem. Ber., 1895, **28**, 1429) and, since Fischer found that this enzyme likewise effects the hydrolysis of lactose, the latter is to be regarded as a β-galactoside. But we shall not go far wrong if we assume, with Bourquelot and Hérissey (C. R., 1903, **137**, 56) and with E. Fischer, that this latter action is not brought about by the same enzyme as hydrolyses β-glucosides, but depends on the presence of an enzyme which decomposes β-galactosides and is hence either identical with, or nearly related to, the lactase occurring in lactose-yeasts. The view that kephir-lactase and emulsin-lactase are different, has been advanced by H. E. and E. F. Armstrong and E. Horton (Proc. Roy. Soc., *B*, 1908, **80**, 321). They assume that the one enzyme is a galacto-lactase and the other a gluco-lactase, the first being taken up by the galactose-residue and the latter by the glucose-residue of milk-sugar.

A preparation which is biologically purer is obtained, according to Pottevin (Ann. Inst. Pasteur, 1903, **17**, 31), from Aspergillus niger, Aspergillus-emulsin hydrolysing only β-glucosides and not β-galactosides or milk-sugar.

Amygdalin is resolved by the enzymes of the almond into glucose, benzaldehyde and hydrocyanic acid, and mandelonitrile glucoside, formed by the action of yeast-enzymes on amygdalin, is also hydrolysed by emulsin into its simplest components (E. Fischer, Chem. Ber., 1895, **28**, 1508). The glucosido-glucose contained in amygdalin is not identical with maltose, since, on the one hand, maltose is not liberated by the action of emulsin (Caldwell and Courtauld, Journ. Chem. Soc., 1907, **91**, 666; Rosenthaler, Arch. der Pharm., 1908, **245**, 684) and, on the other, maltose has no retarding action on the hydrolysis by emulsin (Auld, Journ. Chem. Soc., 1908, **93**, 1276).

It is, therefore, best to indicate by "emulsin" the mixture of glucoside-resolving enzymes and to characterise the preparation according to its origin, a distinction being drawn between Aspergillus-emulsin, almond-emulsin, etc.

For the enzyme-constituents, rational names are then chosen, the results of H. E. and E. F. Armstrong and Horton, Caldwell and Courtauld, and Rosenthaler indicating at least four components of almond-emulsin, namely:

(1) Amygdalase, characterised by the reaction:

$$\underset{\text{Amygdalin}}{C_6H_5\cdot CH(CN)\cdot O\cdot C_6H_{10}O_4\cdot O\cdot C_6H_{11}O_5}+H_2O = \underset{\text{Mandelonitrile glucoside}}{C_6H_5\cdot CH(CN)\cdot O\cdot C_6H_{11}O_5}+C_6H_{12}O_6.$$

(2) A β-glucosidase, which acts on β-glucosides, among them mandelonitrile glucoside:

$$\underset{\text{Mandelonitrile glucoside}}{C_6H_5\cdot CH(CN)\cdot O\cdot C_6H_{11}O_5}+H_2O = \underset{\text{Mandelonitrile}}{C_6H_5\cdot CH(CN)\cdot OH}+\underset{\text{Glucose}}{C_6H_{12}O_6}.$$

(3) A hydroxynitrilase:

$$\underset{\text{Mandelonitrile}}{C_6H_5\cdot CH(CN)\cdot OH} = \underset{\text{Benzaldehyde}}{C_6H_5\cdot CHO} + \underset{\text{Hydrocyanic acid}}{HCN}.$$

In addition to these three substances which take part in the decomposition of amygdalin, the existence in emulsin must be assumed of:

(4) An enzyme which resolves milk-sugar, i.e., a lactase (which Armstrong terms gluco-lactase).

According to Neuberg (Ergeb. der Physiol., 1904, **3**, 446), the conjugated glycuronic acids are also decomposed by emulsin.

The synthetic action of certain components of emulsin is treated more in detail in Chapter VII.

Occurrence. 1. Phanerogams. As well as in almonds, emulsin is found in the leaves of Prunus laurocerasus (where laurocerasin likewise occurs), in the seeds of many of the Rosaceæ, in manihot (Guignard) and in extracts of numerous plants, such as Monotropa, Polygala [Bourquelot, Journ. de Pharm. et Chim., 1904, (5), **30**, 433], Malus communis, Hedera helix, etc. (Hérissey, Thesis, "Recherches sur l'Emulsine," Paris, 1899).

2. Cryptogams. It was discovered simultaneously in Penicillium glaucum by Gérard (Soc. Biol., 1893, **45**, 651) and in Aspergillus niger by Bourquelot, who also detected enzymes capable of attacking glucosides in many other fungi, especially

in the Polyporus species found in wood. Hérissey has found emulsin in many lichens and mosses. Bourquelot has recently observed hydrolysis of otherwise unknown glucosides by emulsin (Arch. der Pharm., 1907, **245**, 172). Fermi and Montesano (Centralbl. f. Bakt., 1894, I, **15**, 722), Gérard (Soc. Biol., 1896, **48**, 44) and Twort (Proc. Roy. Soc., *B*, 1907, **79**, 329) have detected emulsin in bacteria, 27 species out of 44 examined having the property of hydrolysing glucosides.

Worthy of note is the observation of Henry and Auld (Proc. Roy. Soc., *B*, 1905, **76**, 568) that many yeasts also exhibit "emulsin" action.

Animal enzymes closely related to emulsin were found by Gérard (Soc. Biol., 1896, **48**, 44) in the kidneys of the horse and rabbit. In molluscs Bierry and Giaja (Soc. Biol., 1906, **58**, 1038) found enzymes capable of hydrolysing populin and phloridzin; extracts of cross-spiders also resolve amygdalin (Kobert and W. Fischer).

Decompositions of glucosides by animal extracts were also noted by Gonnermann (Pflüg. Arch., 1904, **103**, 225; 1906, **113**, 168) and, more recently by Kobert; according to the latter, extract of placenta hydrolyses amygdalin, arbutin, salicin and helicin.

Preparation: Hérissey (Thesis; compare Bourquelot, Arch. der Pharm., 1907, **245**, 172).

One hundred grams of sweet almonds are steeped for about a minute in boiling water and, after draining, are carefully peeled. They are then ground as finely as possible in a mortar without water, the product obtained being macerated at room-temperature with 200 c.c. of a mixture of equal parts of distilled water and water saturated with chloroform. After about 24 hours, the mass is strained and pressed through a damp cloth. This procedure yields 150–160 c.c. of liquid, to which 10 drops of glacial acetic acid are added to precipitate the casein. The clear filtrate (120–130 c.c.) is added to 500 c.c. of 95% alcohol, the precipitate thus formed being collected on a smooth filter and, after draining, treated with a mixture of equal volumes of alcohol and ether. After drying in a vacuum over sulphuric acid, horny, transparent plates are obtained and, when ground, these give an almost white powder.

Invertase (= α-Fructosidase). Invertin or sucrase owes its name to its property of converting cane-sugar into invert-

sugar (=glucose+fructose). The sphere of action of invertase extends to all synthetic α-methylfructosides; on the other hand, β-methylfructoside, α-glucosides and α-galactosides resist its action. Apart from these synthetic glucosides, gentianose—a trisaccharide from Gentiana lutea—is also attacked by invertase, which resolves it into fructose and gentiobiose. Further, melitriose (raffinose), a trisaccharide occurring in the sugar-beet, is decomposed by invertase, yielding fructose and melibiose.

Occurrence. The distribution of invertase in the yeasts is as well known as important; in the majority of cases, it is accompanied by maltase and, in the lactose-yeasts, by lactase. Invertase occurs alone in only few yeasts, among them being Saccharomyces Marxianus (Fischer, H., 1898, **26**, 75). S. apiculatus contains no invertase.

Of other lower organisms which contain invertase, mention may be made of Fusarium, Streptococcus (Leuconostoc) mesenterioides, Aspergillus oryzae and Monilia candida. A long series of invertase-containing bacteria is also known, especially owing to the investigations of Fermi and Montesano (Centralbl. f. Bakt., 1895, **1**, 482, 542).

With the higher plants, invertase is found especially in the green leaves and young shoots (Kastle and Clark, Amer. Chem. Journ., 1903, **30**, 422), in ripe bananas, in mulberries, in resting and, still more abundantly, germinating pollen, and in wheat and barley embryos; in the crown leaves of Robinia viscosa and pseudacacia, Papaver rhoeas, Rosa species and Bougainvillea bracts. Also in fruits, such as dates, which, when unripe, contain the invertase as an insoluble endo-enzyme, this only becoming soluble when the fruit ripens (Vinson, Journ. Amer. Chem. Soc., 1908, **30**, 1005).

Invertase is found in human intestinal juice, even immediately after birth (Krüger), but not in that of cattle (Fischer and Niebel). Robertson (Edinburgh Med. J., 1894) found it in almost all organs.

Preparation. If yeast-cells are to be extracted with water, it is first of all necessary to kill them, either by treatment for a short time with ether or for a longer time with alcohol (Osborne, H., 1899, **28**, 399), etc., or by heating the dry yeast at 105° (Salkowski) or by plasmolysis (Issaew).

After careful dehydration in a vacuum and subsequent heating, yeast may yield about 12% of its invertase on extraction (Euler and Kullberg, H., 1911, **73**, 94).

Living yeast also gives up invertase to the surrounding liquid —water or sugar solution—but in relatively small quantities. Presumably it is more especially the old cells from which the invertase can be extracted directly.

O'Sullivan and Tompson left top-fermentation beer-yeast for a month at 15° so that it became completely liquid; it was then pressed and the clear solution obtained precipitated with 47% alcohol. The precipitate, after deposition, was dissolved in water and sufficient alcohol added to bring its content in the liquid up to 28%; in this solution the invertase remained dissolved, whilst the majority of the protein substances separated. On raising the alcoholic content of the filtered liquid to 47%, a precipitate was again formed and this was washed with absolute alcohol and dried in a vacuum. The preparation thus obtained was very active, but still not quite pure; it contained about 5% of ash (magnesium and potassium phosphates), which the English investigators regarded as an admixture. Their further purification experiments showed, as had already been indicated by the work of Osborne (H., 1899, **28**, 399) and of Salkowski, that invertase is not a protein. Even the purest preparation contains, besides phosphoric acid, a carbohydrate. Wroblewski regards this as an impurity, as also does Oshima (H., 1902, **36**, 42); the latter came to the conclusion that yeast-gum consists of a substance which contains *d*-mannose and a methyl-pentosan giving fucose on hydrolysis.

Hafner, who carried out a thorough examination of pure invertin (H., 1904, **42**, 1), regards it as by no means disproved that this peculiar carbohydrate always adhering to invertin is an integral constituent of the enzyme. A large part of the phosphorus of invertin preparations is combined organically. The specific activity of the enzyme is not connected with the presence of large nitrogenous groups like the albumoses or peptones; the absence of peptones is also supported by the failure of the biuret action. The nitrogen is probably present in the form of smaller groups, which have, however, not been investigated.

Very active invertase solutions are also obtainable from pure cultures of Aspergillus niger.

The best method for preparing invertase in as pure a form as possible consists in removing protein by lead acetate and kaolin, and in subsequently applying the following diffusion process (Euler and Kullberg, H., 1911, **73**, 335): Bottom fermentation beer-yeast is subjected to autolysis for 3–10 days and then precipitated, as Hudson recommended, with excess of lead acetate; the whole mass is then ground with

kaolin and the liquid pumped off. The lead is precipitated by means of hydrogen sulphide and the filtrate ground several times with kaolin and a little charcoal and filtered. By means of a collodion dialysor, the enzyme solution is freed from the impurities, which diffuse rapidly, and is finally precipitated with alcohol.

Two kilos of pressed yeast, treated in this way, give about 8 grms. of a pure white powder, which is freed from further quantities of nitrogenous impurities by dialysis or diffusion. The preparations are protein-free, and the content of nitrogen varies between 0·3 and 2%. The molecular weight exceeds 25,000.

The activity is $\pm 0° = 10$ minutes, i.e., 0·05 grm. of the preparation, dissolved in 25 c.c. of an 8% cane-sugar solution, reduces the rotation of the cane-sugar to zero in 10 minutes at a temperature of 20°.

The sensitiveness of invertase to temperature (cf. Chapter V) is such that the activity of an invertase solution is diminished by one-half by heating for 30 minutes at 63° (H. Euler and af Ugglas). Its optimum temperature is 53–56°. The influence of the acidity of the solution on the velocity of inversion has been investigated in detail by Sörensen and by Hudson (cf. Chapter IV).

A poisonous action towards invertase is shown by mercury salts and potassium cyanide (and nearly all salts with an alkaline reaction); hydrocyanic acid and chloroform are less harmful, whilst thymol and toluene are without effect.

OTHER ENZYMES WHICH HYDROLYSE GLUCOSIDES

The number of different individuals in this group seems to be very large, but the sphere of action and specificity of the enzymes described are usually very indefinite. Closely related to β- glucosidase is:

Gaultherase or betulase, the specific action of which consists in hydrolysing the glucoside of methyl salicylate (gaultherin). Neither salicin nor amygdalin is attacked by this enzyme.

Occurrence. Exclusively in plants. It was discovered by Schneegans (Arch. der Pharm., 1894, **232**, 437) in the bark of

Betula lenta. At the same time Bourquelot found it in several Polygala and Azalea species, and in Spiraea ulmaria, Monotropa hypopitys and Gaultheria procumbens (C. R., 1896, **123**, 315; J. de Pharm. et Chim., 1896, **3**, 577).

Preparation, according to Bourquelot (loc. cit.): Monotropa plants are ground with sand and the glucoside removed by digesting for half an hour with 95% alcohol. The residue, which contains the enzyme, is quickly dried with alcohol and ether, after which the enzyme can be extracted with water. Cf. Beijerinck (Centrabl. f. Bakt., 1899, II, **5**, 325).

Schützenberger mentioned an enzyme which hydrolyses populin and also phillyrin, a glucoside occurring in the bark of Phillyrea latifolia, but he did not investigate it further.

Sigmund (Monatsh. f. Chemie, 1909, **30**, 77) found an enzyme, which decomposes salicin but does not seem to be identical with emulsin, in certain species of Salix and Populus; he also found one which hydrolyses arbutin in Calluna vulgaris and Vaccinium myrtillus.

W. Sigmund (Monatsh. f. Chemie, 1910, **31**, 657) discovered an enzyme, capable of hydrolysing æsculin, in the seed-coats of the horse-chestnut (Aesculus hippocastanum). It does not appear to be either an amygdalase or a lipase, but is not yet sufficiently defined. Sigmund proposes for it the names, salicase, arbutase and aesculase.

According to T. Weevers (Rec. Trav. bot. Néerland., 1910, **8**) an enzyme which hydrolyses salicin specifically occurs in Salix purpurea and Populus monilifera, and one that hydrolyses arbutin in Vaccinium vitis idaea and Pinus communis.

Bierry and Giaja (Soc. Biol., 1907, **62**, 1117) found, in snails and crustacea, an enzyme which is not identical with "emulsin" but which hydrolyses populin and phloridzin. It remains to be shown that these enzymes are not really general β-glucosidases.

Gease is the name given by Bourquelot and Hérissey (C. R., 1905, **140**, 870) to a specific enzyme from Geum urbanum (Herb Bennett) and rivale which ilberates eugenol from a glucoside contained in these plants.

Elaterase, from Ecballium elaterium hydrolyses elaterin (Berg).

Rhamnase hydrolyses xanthorhamnin, yielding, according to G. and Ch. Tanret (Bull. Soc. Chim., 1899, [iii], **21**, 1065), rhamninose and rhamnetin. Rhamninose is regarded as a trisaccharide, which can be hydrolysed into 2 mols. of rhamnose (methylpentose) and 1 mol. of galactose. The enzyme occurs in Rhamnus infectoria.

Besides these glucoside-enzymes, another series is known which hydrolyse glucosides of one or the other group in a specific manner.

Myrosin decomposes sinigrin or potassium myronate into glucose, potassium hydrogen sulphate and allyl mustard oil (allyl isothiocyanate) according to the equation:

$$\underset{\text{Sinigrin}}{C_{10}H_{18}O_{10}NS_2K} = \underset{\text{Allyl mustard oil}}{C_3H_5\cdot CNS} + \underset{\text{Glucose}}{C_6H_{12}O_6} + KHSO_4$$

Also other sulphur-glucosides occurring in the Cruciferæ are hydrolysed by myrosin; but, according to E. Fischer (Chem. Ber., 1894, **27**, 3483), α- and β-glucosides are not attacked.

Occurrence. The distribution of myrosin has been shown by the investigations of Spatzier (Pringsheim's Jahrb. f. wiss. Bot., 1893, **25**, 39) and, especially, of Guignard (C. R., 1890, **111**, 249 and 920; also Journ. de Bot., 1894, 67 and 85). It is characteristic of the Cruciferæ and certain allied families and is found also in Manihot-species. It is localised in certain cells which are rich in proteins and are dispersed through the tissues. Guignard has isolated mechanically such cells and cell-layers, e.g., the pericycle of Cheiranthus. Roots contain the enzyme mainly in the cork, whilst, in the stem, it is met with especially in the pericycle. Leaves are often very rich in myrosin, which occurs in the young mesophyll.

Poisons and antiseptics: The action of myrosin is prevented by tannin in a concentration of 1% or by salicylic acid in solutions stronger than 1·5%. Chloral in 1% concentration is less harmful, and borax quite harmless. Cf. Reynolds Green, "Soluble Ferments and Fermentation," 1899, p. 154.

Erythrozyme is the name given to an enzyme (Schunck, 1852) which decomposes the ruberythrin or ruberythric acid of madder into alizarin, dihydroxyanthra-

quinone and glucose. This hydrolysis is also effected, although more slowly, by emulsin, with a constituent of which, erythrozyme is closely allied or identical.

Indigo-enzymes. Bréandat found in the leaves of Isatis alpina, an enzyme which decomposes indican, the glucoside of indoxyl, into indoxyl and a sugar (indiglucin). And according to Beijerinck (Malys Jahrb., 1900) an enzyme exists capable of hydrolysing the allied isatan (from Isatis tinctoria).

Lotase from Lotus arabicus decomposes the glucoside lotusin into lotoflavin (1 : 3 : 3′ : 5′—tetrahydroxyflavone), glucose and hydrocyanic acid. According to Dunstan and Henry (Proc. Roy. Soc., 1900, **67**, 224, and 1901, **68**, 374), it is different from emulsin.

Phaseolunatase, investigated by Dunstan, Henry and Auld (Proc. Roy. Soc., *B*, 1907, **79**, 315) seems to be identical with the linamarase of Jorissen and Hairs (Bull. Acad. Roy. Belgique, 1891, **21**, 518) and possibly with maltase.

As mentioned on p. 6, lactase decomposes milk-sugar into d-galactose and d-glucose and, according to E. Fischer, it hydrolyses β-galactosides generally. This action appears to be strictly specific, since neither α- nor β-glucosides are attacked. Maltase and lactase are found in various yeasts (E. Fischer, H., 1898, **26**, 81) and fungi (Bourquelot and Hérissey), and scarcely ever occur together. Eurotiopsis Gayoni forms an exception to this rule, as, according to Laborde (Ann. Inst. Pasteur, 1897, **11**, 1), it attacks both maltose and lactose.

Occurrence. Its existence in lactose-yeasts was assumed by Beijerinck (Centralbl. f. Bakt., 1889, **6**, 44) but was first definitely proved by E. Fischer (Chem. Ber., 1894, **27**, 3481). In the animal organism it does not occur very largely. It is found in both the freshly macerated placenta and in the dry powder. Human intestinal mucus, as well as that of the calf and dog, contain lactase, but only with the young organism. In the intestinal juice of adult man no lactase is found (Hamburger and Hekma, J. de Physiol. et Pathol. gén., 1902, **4**, 805). According to Plimmer (Journ. of

Physiol., 1906, **35**, 20) lactase always occurs in carnivora and omnivora, but with herbivora, with the exception of the rabbit, it is found only in the young animal. Weinland (Zeitschr. f. Biol., 1899, **38**, 606) found this enzyme in the pancreas. Lactase must also occur in all those bacteria which decompose milk-sugar with formation of lactic acid and alcohol, the hexoses, which are formed as intermediate products, resulting from the action of this enzyme; Fuhrmann (Vorlesungen über Bakterienenzyme, p. 96) mentions especially Bacillus acidi laevolactici and Bacterium coli.

Preparation. (Fischer, Chem. Ber., 1894, **27**, 2991 and 3481). Air-dried yeast is carefully ground with powdered glass, the mass being then digested with 20 times its quantity of water for 20 hours at 30-35° and filtered through a Pukall filter. The enzyme solution prepared in this way undoubtedly possessed the property of converting milk-sugar into hexoses, but its action was weaker than that of the aqueous extract of kephir-grains.

Melibiase. Melibiose, a product of the hydrolysis of raffinose, can be further hydrolysed to d-galactose and d-glucose, so that it contains the same components as lactose. This hydrolysis may be effected by an enzyme occurring in certain bottom-yeasts, but not in top-fermentation yeasts. The enzyme is closely allied to maltase and is perhaps to be regarded as a maltase (Fischer, H., 1898, **26**, 81).

PHYTASE

An enzyme which decomposes phytin or inositolhexaphosphoric acid, $C_6H_6[OPO(OH)_2]_6$, into inositol and phosphoric acid, was obtained by N. Suzuki, Yoshimura and Takaishi (Bull. Coll. Agric., Tokyo, 1907, **7**, 503) from rice- and wheat-bran. The preparation itself was free from phosphorus and hydrolysed neither amylose nor proteins. Presumably the enzyme, like phytin, is widespread in the vegetable kingdom. According to McCollum and Hart (Journ. of Biol. Chem., 1908, **4**, 497), phytase is also contained in the liver and blood, but not in muscle- or kidney-extract. Quite recently, A. W. Dox and Ross Golden (Journ. of Biol. Chem., 1911, **10**, 183) have detected phytase in lower fungi.

HEXOSEPHOSPHATASE

According to Harden and Young (Proc. Roy. Soc., *B*, 1910, **82**, 327), this enzyme, which occurs in pressed yeast juice and also in yeast dried at room-temperature, separates the phosphoric acid from hexosephosphate. See later under "zymase."

PECTASE

It is undoubtedly most rational to employ the name pectases for those enzymes which convert pectoses into pectin and pectinic acids. The reaction, which yields also arabinose, consists certainly of a hydrolysis, but the details of the chemical changes occurring are unknown. The pectinic acids formed exist in the plants as calcium salts. The work of Mangin (C. R., 1888–1893) and of Devaux (Soc. phys. nat. de Bordeaux, **3**) can only be mentioned here. The enzyme here termed pectase was obtained from malt-extract by Bourquelot and Hérissey (C. R., 1898, **127**, 191; 1899, **128**, 1241), who called it pectinase; according to the general principle of naming the enzyme after the substrate, this should be altered to pectase.

PECTINASE

By the term pectinase should be indicated the enzyme which coagulates dissolved pectin-substances, e.g., in fruit-juices, in presence of lime, to gelatinous calcium salts of the feebly acid pectinic acids. Here also there is as yet no explanation of the chemical reaction taking place. A high concentration of acid retards or completely prevents coagulation, which, in the case of acid fruit-juices, proceeds only after neutralization with lime. The velocity of the reaction is conditioned by a certain equilibrium between the enzyme and the concentrations of the acid and calcium salts. Without the action of the enzyme, the lime is unable to coagulate pectins to calcium pectates; soluble calcium salts can, indeed, induce pectins to coagulate, but, in this case another product is formed, namely a pectinate soluble in 0·2% hydrochloric acid. Calcium pectate, however, yields insoluble pectinic acid with 0·2% hydrochloric acid. It

should be noted that Bertrand and Mallèvre, to whom is due a thorough investigation of this enzyme (C. R., 1894, **119**, 1012; 1895, **120**, 110, and **121**, 726), named the latter pectase.

Occurrence. It is found in nearly all plants, especially in young, quickly-growing organs, shoots, leaves, roots, and fruit. In great abundance and in an extremely active condition, the enzyme appears in the extract of clover or lucerne, and also in the leaves of the potato plant, rape, etc.

CARBAMASES (Proteinases)

Three proteolytic enzymes or groups of enzymes are distinguished: the pepsin of the gastric secretion, the trypsin of the pancreas and the erepsin of the intestinal mucus. It is very probable that these three substances are not individuals, but rather mixtures of enzymes, which yet act specifically on certain protein complexes. These three enzyme-groups can, however, be readily differentiated according to their origin. More difficult is the division of the vegetable proteinases, where classification is not possible according to either the localisation of the enzymes or the media in which they act. Hence, a distinction between vegetable pepsins and vegetable trypsins can hardly be drawn. The only classification at present apparent depends, on the one hand, on a separation of the peptases from the true proteinases, and, on the other, on a limitation of the pepsinases, for which the absence of the lower hydrolytic products is characteristic. It is, however, doubtful whether trypsin itself carries the hydrolysis further than pepsin does, or whether it owes this property to an accompanying peptase. In any case, a strict division of the proteinases is at present difficult.

Pepsin: Pepsinases

The decomposition of proteins effected by pepsin extends, so far as is known, to all proteins; the products formed consist of albumoses and peptones, so that the hydrolysis is incomplete, lower polypeptides and amino-acids not, as a rule, appearing (Abderhalden and Rostocki, H., 1905, **44**, 265).

Occurrence: In the gastric juice of all the vertebrates examined, with the exception of certain fishes. The pepsin of Brunner's glands is closely allied to gastric pepsin. In the gastric mucous membrane, pepsin occurs not in an active form but as a pro-enzyme (pepsinogen). It exists with new-born children and, with certain herbivorous animals, even in the fœtal state, in the mucous membrane, but is not present at birth in those carnivora which have been examined, namely, the dog and the cat. Proteolytic enzymes, similar to pepsin and active in acid solution, have also been found in several invertebrates, but, with some animals at least, these enzymes are not identical with ordinary pepsin.

Preparation: Very active and stable pepsin-solutions are obtained by extracting the gastric mucous membrane with glycerol; the pepsin, together with protein, may be precipitated from the extract by means of alcohol. A considerable amount of pepsin can also be extracted from this membrane by acidified water. The best starting material is, however, pure gastric juice, which can be prepared by Pawlow's well-known method from gastric fistulæ.

Attempts at the purification of pepsin have, up to the present, led to no final result, but Pawlow's gastric juice is the best material to work on for this purpose. Such gastric juice, on freezing, yields a solid product, which Mme. Schumoff-Simanowski has called "granular pepsin" (Körniges Pepsin).

The preparation of the enzyme in a pure state has been worked at mainly by Nencki and Sieber (H., 1901, **32**, 231; **33**, 291) and by Pekelharing (H., 1902, **35**, 8).

According to Pekelharing's method, Pawlow's gastric juice is dialysed for 20 hours at 0°, the pepsin thus being deposited in transparent granules at the bottom of the dialyser. The turbid liquid is centrifuged and the colourless residue pressed and dried; in Pekelharing's opinion, this represents pure pepsin.

The percentage composition of the enzyme is found to be:

	C	H	N	S	Cl	P	Fe	Ash
Nencki and Sieber:	51·26	6·74	14·33	1·5	0·48	small	variable	0·57
Pekelharing:	51·99	7·07	14·44	1·63	0·49	0	+	0·1

According to Nencki and Sieber, pepsin is combined with lecithin. When boiled with acids, pepsin yields an

albumose and a nucleo-proteid, which then yields purine-bases (Pekelharing found xanthine) and pentoses (Friedenthal, Engelmann's Arch. f. Physiol., 1900, **24**, 181).

They regarded the pepsin molecule as performing (by three different groups) the three functions of the gastric juice: protein digestion, clotting and plastein formation, a view with which Pekelharing agreed.

The precipitate formed when gastric juice is coagulated by heat contains, according to Pekelharing, an acid — pepsinic acid — which has the percentage composition: C, 50·79; H, 7·0; N, 14·44 and S,1·08 and gives Millon's and the biuret reactions.

Formaldehyde does not act on "pepsin," and this fact caused Bliss and Novy to doubt the protein character of pepsin. Further, as was shown by Nencki and Sieber and also by Pekelharing, it is possible to prepare pepsin solutions which vigorously digest proteins but do not show the protein reactions; indeed, this was shown to be the case as early as 1885 by Sundberg (H., 1885, **9**, 319; cf. Brücke, Wiener Sitz.-Ber., 1861, **43**, 601).

The preparation of a protein-free pepsin solution, which has an energetic digestive action but does not produce clotting, was described by Schrumpf (Hofm. Beitr., 1905, **6**, 396). The mucous membrane was separated from a fresh pig's stomach and was then ground with kieselguhr and pressed under a high pressure. The pressed juice was clarified by means of a Kitasato filter-candle and was found to remain clear on addition of uranyl acetate, ammonium sulphate, etc.

Herlitzka (Atti Real. Accad. Lincei., 1904, [v], **13**, ii, 51) has deduced a proof for the opposite view—that pepsin is a true protein—from the fact that, in absence of hydrochloric acid, pepsin gradually loses its activity, peptones appearing at the same time. These may, however, result from admixtures, so that the investigation proves nothing concerning the chemical nature of pepsin.

As regards the individuality of pepsin, it has already been indicated that the same molecule, by means of other side-chains, causes rennet-action and plastein-formation, and this hypothesis is not in discord with the facts at present known (cf. Jacoby, Biochem. Z., 1906, **1**, 53). On the other hand, justifiable

objections have been raised against Pawlow's assumption that the rennet-action is a reversal of the pepsin-action (Bang, H., 1904, **43**, 358; Schmidt-Nielsen, H., 1906, **48**, 92; especially Hammarsten, H., 1908, **56**, 18).

From its chemical characters, pepsin appears to be an acid, and this view is sustained by a number of different observations by various investigators. Jacoby (Biochem. Z., 1907, **4**, 471) found that pepsin, and also rennin, are soluble in alkali, and adsorption experiments described by Michaelis indicate marked adsorption by basic media. This view is also supported by the observation that pepsin migrates to the anode.

Pepsin is inactive in an alkaline medium, but on acidification it recovers its activity more or less completely according to the nature and duration of the previous alkalinity (Tichomirow, H., 1908, **55**, 107).

Pepsin exerts its optimal activity at 40° and its stability is greater in acid than in neutral solutions and is further increased by the presence of salts.

Trypsin: Tryptases

The enzyme of the pancreas, which is active in alkaline or neutral solution, resolves the proteins into simple polypeptides, these being to some extent further broken down. It does not decompose all dipeptides, but presumably only those combinations occurring in the organism (cf. Chapter VIII). On the other hand, it can be stated that only those polypeptides are attacked which contain naturally-occurring, optically active amino-acids. Acid-amides, hippuric acid, etc., are not attacked (Gulewitsch, H., 1899, **27**, 540; Schwarzschild, Hofm. Beitr., 1903, **4**, 155; Fischer and Bergell, Chem. Ber., 1903, **36**, 2592 and 1904, **37**, 3103). With nucleo-proteins the protein component is separated from the nucleic acid and decomposed further. From certain nucleo-proteins, the phosphoric acid is liberated (Bayliss, Arch. Sci. Biol. St. Petersburg, 1904, **11**, Suppl., 281).

Not at all improbable is the assumption of Schaeffer and Terroine (J. de Physiol. et Pathol. gén., 1910) that the trypsin of the pancreas is accompanied by an erepsin which

attacks directly (without kinase) all substances split off by the gastric juice.

Occurrence. Trypsin occurs in the pancreatic juice and in the tissues of the pancreatic glands as a pro-enzyme, which is transformed by specific activators or kinases into the active condition.

Trypsins or tryptases have been found in all the vertebrata tested for them. Hedin (Journ. of Physiol., 1903, **30**, 155 and 195) discovered a tryptic enzyme in normal blood-serum; it acts in an alkaline medium and decomposes casein, gelatine, and coagulated serum-albumen, but globulins and coagulated egg-albumen are not attacked. Besides in the pancreas, animal tryptases are found in the urine, and in the spleen and other organs; also in hens' eggs.

Enzymes which must be termed tryptases are contained in the leucocytes and, as Jochmann showed, not only in leucemic, but also in normal, leucocytes. This occurrence is limited, so far as is known to man, monkeys, and dogs (Erben, Münch. Med. Wochens., 1906 and 1907; Jochmann and Lockemann, Hofm. Beitr., 1908, **11**, 449). The tryptase of the leucocytes is less sensitive to heat than that of the pancreas; its optimal temperature is 55°.

Tryptases have also been found in insects, protozoa, sponges, worms, and molluscs.

Preparation. Active but impure solutions may be obtained by extracting finely-chopped pancreatic glands with chloroform-water. The best material is pancreatic juice obtained by Pawlow's method and activated by intestinal secretion.

The purification of trypsin has been recently attempted, especially by Mays (H., 1903, **38**, 428 and 1906, **49**, 124 and 188). He obtained a relatively very pure and active preparation by salting-out the trypsin solution with sodium chloride and magnesium sulphate; but his investigations indicate little concerning the chemical nature of trypsin. It is not a nucleo-protein and does not give the biuret reaction (Mays, Schwarzschild).

In aqueous solution, it is very labile and sensitive to heat, and, indeed, more so in alkaline than in neutral solution. Substrate and decomposition products exert a considerable protecting action (Bayliss, Vernon), so that the optimal temperature is given as 40°. Trypsinogen is less sensitive to heat than trypsin. Acids denature it even in low concentrations. In contrast to pepsin, which dissolves in alkali, trypsin is

soluble in acids. In the electric field, it behaves as an amphoteric electrolyte, that is, it migrates either to the anode or to the cathode according to the reaction of the solution. It is affected but slightly by chloroform or thymol.

Pollak (Hofm. Beitr., 1905, **6**, 95) characterises glutinase as a separate enzyme, owing to its resistivity towards acids. It acts on glue, but not on certain other proteins.

On the other hand, Ascoli and Neppi (H., 1908, **56**, 135) have established the fact that the specificity of glutinase towards glue is only apparent and is due to the different proteins being influenced differently by activators and paralysers

Erepsin

See Cohnheim (H., 1901, **33**, 651; 1902, **35**, 134; 1902, **36**, 13; 1906, **49**, 64). This enzyme decomposes albumoses, polypeptides, peptones and protamines completely into amino-acids. With the exception of casein, proteins are not attacked, but nucleic acids are decomposed (Nagayama, H., 1904, **41**, 348).

Occurrence: in the intestinal juice and the intestinal mucous membrane, the latter being the richer in the enzyme. It appears, especially after Vernon's investigations (Journ. of Physiol., 1904, **32**, 33) and those of Abderhalden, to be the most widely distributed proteolytic enzyme; it has been detected in nearly all the animals examined for its presence.

Preparation. According to Cohnheim, it can be prepared from the pressed juice of the intestinal mucous membrane; also extraction of the latter with glycerol or water yields very active solutions. From the aqueous solution, the enzyme is salted out with ammonium sulphate. Pressed yeast juice likewise contains much erepsin (Abderhalden).

The optimal temperature is about 38° (in alkaline solution).

PROTEOLYTIC ENZYMES OF PLANTS

Proteinases is the name given to those enzymes which break down the true proteins. The decomposition appears to proceed as far as the albumoses and peptones. It is seldom that proteinases occur alone in the organs of plants where

proteins are decomposed; they are almost always accompanied by peptases, which correspond with the erepsins of the animal body. Vines (Annals of Bot., 1904, **18**, 289; 1905, **19**, 149, 171; 1906, **20**, 113; 1908, **22**, 103; 1909, **23**, 1) to whom we owe a thorough investigation of the vegetable proteinases has, indeed, recently found both enzymes in the seeds of Cannabis sativa.

Occurrence. Proteinases occur especially in germinating and ungerminated seeds and more abundantly in oil-bearing than in starch-containing seeds—particularly in Cannabis sativa, Sinapis, Ricinus and Linum; further, in certain juicy fruits (Ficus carica) and leaves (Agave). They are generally found in insectiverous plants. Among those which have been thoroughly investigated are

Bromelin in acid banana-juice (Chittenden, Journ. of Physiol., 1894, **15**, 249); according to Caldwell (Bot. Gaz., 1905, **39**, 407), this consists of two components, a pepsinase and a tryptase; and

Papain or papayotin in the juice of the fruit, leaves and stem of the papaw tree (Carica papaya). These two enzymes are very closely allied if not absolutely identical. Animal proteins, such as fibrin, are also hydrolysed by bromelin (Chittenden). From the mixture of proteolytic enzymes in oily seeds, Vines isolated a fibrin-digesting proteinase. A similar action is exhibited by the papain of Carica which readily digests fibrin. After digestion of fibrin with Carica-sap and with an enzyme similar to papain and obtained from Bacillus fluorescens liquefaciens, Emmerling (Chem. Ber., 1902, **35**, 695) found in addition to albumoses and peptones, also various amino-acids, such as leucine, tyrosine, etc. With "papayotin Merck," Kutscher and Lohmann (H., 1905, **46**, 383) obtained analogous results. According to Abderhalden and Teruuchi, H., 1906, **49**, 21), papain splits glycyl-l-tyrosine; this also indicates the simultaneous occurrence of proteinases and peptases, unless, indeed, papain is regarded as a tryptase with a wider sphere of action.

Vegetable proteases accompany malt-diastase and taka-diastase. These proteinases are extractable by alcohol in a remarkable manner (Vines, Annals of Bot., 1910, **24**, 213).

The statements made concerning the acidity or alkalinity of the medium in which vegetable proteinases exhibit their maximal activity differ considerably (cf. Reynolds Green, Vines, Emmerling, Weis, and others). This dis-

agreement depends partly on qualitative and quantitative differences between the substrates employed and partly on varying composition of the enzyme preparations. The peptase present will always act best in neutral or faintly alkaline solutions and the proteinases, on the other hand, in acid ones. Proteinases occur abundantly in fungi and they can be extracted from the mycelia by water or glycerol.

As extracellular enzymes, proteinases occur in insectivorous plants. In these they are liberated from zymogens in certain glands under the stimulative action of nitrogenous substances. With Nepenthes, the enzyme is distributed in the antiseptic, almost protein-free sap of the leaf-pitchers. This acid sap decomposes proteins not merely into albumoses, but also into amino-acids (Vines, Annals of Bot., 1897, **11**, 563). But Abderhalden found that glycyl-l-tyrosine is not attacked. Hence uncertainty still prevails regarding the nature of the enzyme or enzymes of Nepenthes. The leaf-glands of Dionaea and Drosera and the leaf-edge of Pinguicula yield acid, mucilaginous secretions which attack proteins of cartilaginous and glutinous tissues. Extracellular proteinases also appear to be produced by bacteria, especially by those species which are capable of liquefying gelatine.

Another group of fungus-proteinases consists of typical endoenzymes; noteworthy among these is the

Endotryptase of yeast (Hahn and Geret). So far as is known, its action extends to all proteins (gelatine, fibrin, casein, egg-albumin), which it resolves into amino-acids. It cannot be extracted from yeast by water, and is obtainable only by Buchner's method.

Unlike papain, endotryptase is most active in acid solution. Its temperature-optimum is 40–45°.

β-Proteases. Papayotin, Hahn and Geret's endotryptase and the enzymes which effect a partial decomposition of the protamines and complicated proteins in faintly acid solution, are classed together by Takemura as β-proteases. The action of pepsin on protamines would thus be attributable to the presence of a β-protease.

Peptases are those enzymes which decompose albumoses, peptones and polypeptides into amino-acids. They generally accompany the proteinases and, as has been shown by Vines

(Annals of Bot., 1906, **20**, 113), Abderhalden and Schittenhelm (H., 1906, **49**, 26) and Euler (H., 1907, **51**, 244), they occur abundantly in the seeds of lupins, rape, peas and maize. Abderhalden and his collaborators found it also in pressed yeast juice.

According to certain investigators, among them Weis (H., 1900, **31**, 79) and Vines (Annals of Bot., 1903, **17**, 237 and 1904, **18**, 289), the most rapid hydrolysis takes place in faintly acid solution, whilst others, e.g., Windisch, assert that it occurs in a slightly alkaline medium.

NUCLEASES

The decomposition of the nucleo-proteins begins with their resolution into nucleic acids and protein-components, a reaction which is brought about by pepsin or trypsin. The further division of the nucleic acids is not, however, produced by the true proteinases (Sachs; Abderhalden and Schittenhelm, H., 1906, **47**, 452), but by a special group of enzymes, the nucleases. These, therefore, resolve the nucleic acids into their constituents, purine or pyrimidine bases (adenine, guanine, cytosine, thymin), pentoses, and phosphoric acid.

That the decomposition of nuclein, and the digestion of Buchner's pressed yeast juice are enzymic in character has been shown by Salkowski (H., 1889, **13**, 506) and Hahn and Geret (Chem. Ber., 1898, **31**, 2335) respectively, and more recent researches prove that a number of enzymes act on the nucleic acids. These enzymes may be divided into a number of groups (cf. B. Bloch, Biochem. Centralbl., 1907, **5**, 561).

1. Nuclease, which liberates the phosphoric acid from the nucleic acid molecule.
2. Hydrolytic enzymes, which split off ammonia from the aminopurines and replace the amino-group by hydroxyl.
3. Oxidising enzymes which oxidise hydroxypurines to uric acid.
4. Uric acid oxydases.

According to Sachs (H., 1905, **46**, 337), pure trypsin has no action at all on nucleic acids. Decomposition of nucleic

acids takes place only in fresh pancreas extracts, which exert but slight proteolytic action.

Occurrence. Very frequent in animal organs, e.g., in the spleen, liver (Jones), and in the pancreatic and thymus glands (Kutscher, H., 1901, **34**, 114). It performs an important function in many organs of plants—more particularly in germinating seeds—in synthesizing and decomposing nucleo-proteins (Zaleski, Bot. Ber., 1907, **25**, 349). Worthy of note also are the nucleases of higher (Kikkoji, H., 1907, **51**, 201) and lower fungi (Iwanoff, H., 1903, **39**, 31), especially those of yeast.

P. A. Levene and Medigreceanu (Journ. of Biol. Chem., 1911, **9**, 389) have recently proposed the following classification and nomenclature.

Nucleinases resolve the nucleic acid molecule into nucleotides.

Nucleotidases decompose nucleotides into phosphoric acid and a carbohydrate-base complex (nucleoside).

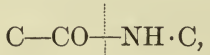
Nucleosidases hydrolyse nucleosides into ribose and purine bases.

Arginase (Kossel and Dakin, H., 1904, **41**, 321)

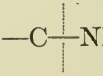
This enzyme hydrolyses arginine specifically into urea and ornithine according to the following equation:

$$NH_2\cdot C(=NH)\cdot NH\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH(NH_2)\cdot COOH + H_2O = NH_2\cdot CO\cdot NH_2 + NH_2\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH(NH_2)\cdot COOH.$$

Whilst trypsin hydrolysis may be indicated by the scheme:



the action of arginase is represented by



Occurrence. In the liver, kidneys, thymus, and intestinal mucous membrane of the calf and also in the muscles of the dog and

the lymphatic glands of cattle. Shiga (H., 1904, **42**, 502) found arginase also in yeast.

It can be extracted from the organs by means of water.

Urease

This enzyme decomposes urea into carbon dioxide and ammonia.

Occurrence. According to Leube (Virch. Arch., 1885, **100**, 540), urease occurs in Micrococcus ureae, from which it passes readily into the surrounding liquid. In cystitic urine the enzyme occurs only when fungi capable of decomposing urea are present.

Lea (Journ. of Physiol., 1885, **6**, 136) has attempted to prepare urease in a pure state; by treatment of Micrococcus ureae with alcohol, extraction of the precipitate with water and repeated reprecipitation with alcohol, a powder is obtained which yields a clear aqueous solution but contains protein. As later work has shown, urease is tenaciously retained by living protoplasm. Moll (Hofm. Beitr., 1902, **2**, 344) also obtained the enzyme in a similar manner and from the same material; it readily undergoes decomposition. Schittenhelm obtained an enzyme-solution (free from purine-bodies) from the kidneys and Kikkoji found urease in a pileate fungus.

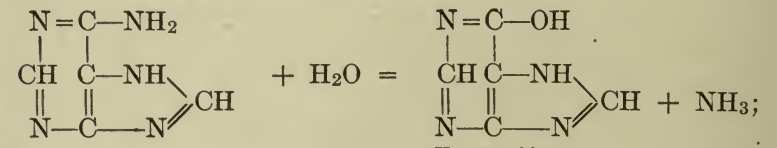
AMIDASES (DESAMIDASES)

Decomposition of the proteins by trypsin and erepsin ceases when the amino-acids are reached, but the desamidases attack the latter, decomposing them into ammonia and hydroxy-acids. Such simple desamination, as was discovered by H. Pringsheim (Biochem. Z, 1908, **12**, 15), takes place by virtue of an enzyme present in acetone-yeast. Similar action is exhibited by permanent preparations of Aspergillus niger (Shibata, Hofm. Beitr., 1904, **5**, 384). It has been shown by Pringsheim, Abderhalden and Schittenhelm that the amidases of yeast do not pass into the press-juice. Of biological importance is the detection of amidases in higher plants (Butkewitsch, H., 1909, **63**, 103; cf. Kiesel, H., 1910, **65**, 283).

The so-called "alcoholic fermentation of the amino-acids" —discovered by F. Ehrlich—consists of a combination of two reactions, namely the desamination of the amino-acids to the corresponding hydroxy-acids and the subsequent loss of carbon dioxide from the latter; the total reaction can hence be formulated thus:

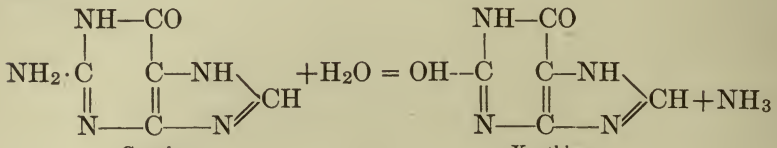
$$R \cdot CH(NH_2) \cdot CO_2H + H_2O = R \cdot CH_2 \cdot OH + CO_2 + NH_3.$$

Reactions similar in principle to those brought about by amidases are caused by the enzymes known as guanase and adenase. These convert guanine and adenine into the corresponding hydroxy-derivatives:



Adenine Hypoxanthine

and



Guanine Xanthine

Concerning the individuality and mode of action of these enzymes of the purine-bases, various views have been expressed by the different investigators who have studied them (Schittenhelm, H., 1904, **42**, 251; 1905, **43**, 228; **45**, 121, 152; **46**, 354; Jones and his collaborators, H., 1904, **42**, 35, 343; 1905, **44**, 1; **45**, 84; 1906, **48**, 110; and also Burian, H., 1905, **43**, 494). Even if Schittenhelm's assumption that the two enzymes are identical is not correct, they are certainly very similar.

Occurrence. Guanase and adenase have been found in the pancreas, spleen, lungs, and liver of the child, pig (not in the spleen), cattle, and also in numerous other organs.

Preparation. (Schittenhelm, H., 1904, **42**, 251). One or two spleens are extracted with about 2 litres of water for 12 hours and the enzyme then precipitated with

ammonium sulphate. The precipitate is dissolved in water and freed from ammonium sulphate by dialysis.

Nitrilase

According to Rosenthaler (Biochem. Z., 1909, **19**, 186; 1910, **28**, 408), the decomposition of amygdalin is effected by three enzymes—an amygdalase, a β-glucosidase and an hydroxynitrilase. The last of these exerts a catalytic influence on the reaction.

$$C_6H_5 \cdot CH\begin{matrix}OH\\ \diagup\\ \diagdown\\ CN\end{matrix} + H_2O = C_6H_5 \cdot CHO + HCN,$$

and, in Rosenthaler's opinion, its action is solely hydrolytic, i.e., it can be separated from the synthesising constituent of emulsin; in Chapter VII of this book, hydroxynitrilase (oxynitrilase) will be considered, together with the corresponding synthesising enzyme, in greater detail.

COAGULATING ENZYMES

Chymosin and parachymosin are those enzymes which effect the clotting of milk, the casein of the latter being changed in some way, as yet unknown. The two enzymes are distinguished according to their place of origin. Chymosin which has been long known and is the enzyme of the calf's stomach, is, according to Bang (Deutsch. med. Wochens., 1899, and Pflüg., Arch., 1900, **79**, 425), to be distinguished from the coagulating constituent of the human stomach, namely, parachymosin. The chemistry of rennet-action is still not clear. With solutions containing pure casein and also rennet in as pure a state as possible, the clear whey obtained after coagulation contains a very small proportion of a protein—whey-albumin—with only 13·2% of nitrogen. The bulk of the casein is precipitated as a substance, paracasein, very similar to casein itself. Whether decomposition of the casein occurs is still uncertain (cf. Hammarsten, Text-book of Physiological Chemistry, 4th Edition, 1906, 442).

Occurrence. In the gastric juice, the gastric mucous membrane, the pancreatic juice, the placenta, and certain other organs of many

different animals The enzyme often occurs in an inactive form as the so-called prochymosin (Edmunds, Journ. of Physiol., 1895, **19**, 466; Vernon, Journ. of Physiol., 1901, **27**, 174), owing mainly to the lack of an activator.

The activation of prochymosin can be effected instantly by an acid and, after activation, the rennet is active in both neutral and alkaline solutions.

Preparation. (Hammarsten, H., 1908, **56**, 18). This author has recently described the preparation of chymosin from the stomach of the calf, horse, hen and pike. The first-named material is treated as follows:

The innermost coat of the stomach is separated from the intestines and from the three other stomachs, cut along the small curvature and freed from contents by rinsing out with water. The pylorus portion is then cut away together with at least 3–5 c.m. of the large fold of the fundus portion. (The pylorus part yields a more mucilaginous infusion and is, at the same time, less rich in enzymes, than is the fundus.) The remainder of the stomach is stretched out and thoroughly washed. The glandular layer is then scraped from the two sides of a fold with the edge of a watch-glass, weighed and introduced into 0·1–0·2% hydrochloric acid, 10–20 c.c. of the latter being taken per grm. of the glandular matter. The acid is allowed to act, with repeated shaking, for 12–24 hours at a low temperature—somewhat above 0°—the liquid being then filtered. The infusion is neutralised and, if found to exert a vigorous coagulating action, is precipitated with magnesium carbonate.

One grm. of magnesium carbonate is added to each 100 c.c. of the extract, which is shaken and quickly filtered; it can then be tested for the presence of pepsin (see Appendix: Practical Methods). If the filtrate still contains much of this enzyme, it is again treated with magnesium carbonate, and this procedure is repeated until a filtrate is obtained which readily causes clotting of milk but has only a slight action on fibrin.

Another process which Hammarsten gave for the preparation of chymosin consists in freeing it from the greater part of the pepsin by means of magnesium carbonate and precipitating the chymosin with lead acetate. The lead is removed from the precipitate by sulphuric acid and the acid filtrate shaken

with an alcoholic solution of cholesterol or stearic acid and a little ether, so that the precipitated cholesterol or stearic acid carries down the enzyme with it. If now the precipitate is shaken with water and freed from the precipitant by treatment with ether, a moderately pure chymosin solution is obtained. The purest clotting enzyme obtained in this way did not give the ordinary reactions for proteins.

Two different views are held as to the identity of chymosin and pepsin. According to the one, which has been advanced by Pawlow (H., 1904, **42**, 415), Sawjalow (H., 1905, **46**, 307), Sawitsch (H., 1908, **55**, 84) and Gewin (H., 1907, **54**, 32), both actions result from one and the same enzyme, Sawjalow and Gewin regarding the clotting of milk as the commencement of pepsin-digestion. On the other hand, Nencki and Sieber (H., 1901, **32**, 291), Pekelharing (H., 1902, **35**, 8), Schmidt-Nielsen (H., 1906, **48**, 92), Taylor (Journ. of Biol. Chem., 1909, **5**, 399), and especially Hammarsten (H., 1908, **56**, 18) are of opinion that the two enzymes are not identical but of different kinds.

In addition to the cholesterol method described above, Hammarsten has recently given a second method allowing of the separation of chymosin-action from that of pepsin. In principle this method consists in heating the acid enzyme-solution to 40° or a rather higher temperature. In this way the calf-chymosin is destroyed more rapidly than the pepsin, so that after some time a solution is obtained which no longer exerts a clotting action, but still digests proteins (loc. cit., p. 61).

Still more recently (H., 1911, **74**, 142), Hammarsten has succeeded in preparing pepsin-free chymosin solutions by mixing an acid infusion of calf's stomach and a neutral alkali caseinate solution in such proportions that the casein at first separating just redissolves. To the acid casein solution thus obtained, decinormal sodium hydroxide solution is added in sufficient quantity to produce an abundant precipitation of casein and to allow of ready filtration while the reaction still remains strongly acid. Both the enzymes are carried down by the precipitated casein, but the pepsin in much larger quantity than the chymosin. The filtrate therefore contains a relatively high proportion of chymosin.

Chymosin exhibits remarkable activity, one part of the mucous membrane of the calf's stomach being sufficient to clot 250,000 parts of milk. Purified chymosin coagulates 24,000,000 (Hammarsten) or 30,000,000 (Fuld) times its weight of milk.

Chymosin causes clotting only in acid solutions; hydrochloric acid is the most favourable to its action and after this come nitric, lactic, acetic, sulphuric and phosphoric acids. Its optimum temperature is 37–39°.

Chymosin is injured by chloroform (Benjamin), but not by hydrocyanic acid (Fuld and Spiro).

Milk-clotting Enzyme in Plants (Cynarase)

A large number of plants, such as Pinguicula vulgaris, Galium verum, artichokes, etc., possess the property of rendering milk ropy.

Whether the chemical change underlying this coagulation is or is not the same as that produced by the action of chymosin, is unknown.

The vegetable chymases must, however, be quite different from the animal enzymes. According to Chodat and Rouge (Centralbl. f. Bakt., 1906, II, **16**, 1), the sykochymase from Ficus carica investigated by them acts in absence of calcium salts. Its optimal temperature is 75–80°.

Further, as has been shown by Bruschi (Atti Real. Accad. Lincei, 1907, [V], **16**, ii, 360) and especially by Gerber (C. R., 1907, **145**, 689; 1908, **146**, 1111; **147**, 601, 1320; 1909, **148**, 497, 992; **149**, 137, 737; 1910, **150**, 1202, 1357), the phytochymases exhibit great differences among themselves. Gerber distinguishes these enzymes according to the amounts of lime they require for their action, and he has further shown that some phyto-chymases coagulate raw milk, while others coagulate boiled milk the more readily.

Occurrence. In addition to the plants mentioned above, the following also exert a coagulating action on milk: Lolium perenne, Anthriscus vulgaris, Plantago lanceolata, Lamium amplexicaule and hybridum, Philadelphus coronarius, Geranium molle, Capsella bursa pas-

toris, Ranunculus bulbosus, Medicago lupulina, Centaurea scabiosa, etc. Reynolds Green (Proc. Roy. Soc., 1890, **48**, 370) found the enzyme in the germinating seeds of Ricinus communis in the form of a zymogen, which is activated by dilute acids. This enzyme is also contained in numerous other seeds, for example, in those of Datura, Pisum, and Lupinus hirsutus. Weis (H., 1900, **31**, 79) found a clotting enzyme in malt. The coagulating action of the fig, Ficus carica, on milk was known to the ancient Greeks. See also Gerber (C. R., 1909, **148**, 992).

Lastly, coagulating enzymes have also been detected in many lower fungi, among others Fuligo varians. C. Gerber has recently examined 86 species and sub-species of Basidiomycetes, and has found them to contain, in general, an "oxyphile" and a "calciphile" coagulating enzyme.

Thrombin, Fibrin-ferment (Alexander Schmidt, 1872)

Thrombin causes blood to coagulate by converting dissolved fibrinogen into insoluble fibrin.

The change taking place is probably as follows: When the blood leaves the body, one of its constituents (possibly the leucocytes) gives rise to a pro-enzyme, which is converted into the active enzyme under the influence of the calcium salts. This enzyme, without further action of calcium salts, then transforms the fibrinogen into insoluble fibrin.

It is best prepared from blood-serum or defibrinated blood by precipitation with 15–20 volumes of alcohol, which separates the proteins at the same time. If the precipitate is then extracted with water, part of the protein remains undissolved, whilst the thrombin passes into solution. According to Hammarsten's method (Pflüg. Arch., 1878, **18**, 38) the globulins are first precipitated by magnesium sulphate; the liquid is then diluted with water and sodium hydroxide solution added so as to precipitate magnesium hydroxide, which is accompanied by a considerable amount of adsorbed fibrin-ferment. Pekelharing dialyses the filtrate from the precipitate given by magnesium sulphate. From the muscles of birds, Fuld obtained thrombin by extraction with 0·8% sodium chloride solution.

According to Shigeji and Higuchi, the placenta contains a fibrin-enzyme, which can be extracted by means of water or physiological salt solution.

Pekelharing regards thrombin as the lime compound of pro-thrombin, the nucleoprotein which occurs in blood-plasma and which he attempted to prepare in a pure condition (Verh. d. k. Akad. v. Wetenschappen te Amsterdam, 1892, II, **1**, No. 3; and Zentralbl. f. Physiol., 1895, **9**, 102). The existence of this nucleoprotein in blood-serum has been established, but its composition has not yet been investigated since the quantity of it in blood is very small. In a concentrated solution of the enzyme, which contained 0·417% of organic matter and 0·166% of inorganic matter, Hammarsten found only 0·005% of nuclein. The experimental results obtained by Huiskamp (H., 1901, **32**, 145), led this investigator to dispute Pekelharing's views. Huiskamp found that, in presence of calcium salts, both the nucleohistone and the other nucleoprotein of the thymus glands—two essentially different substances—acted on fibrinogen in the same way as thrombin; for the calcium nucleoprotein he gave the following percentage composition: C, 49·82; H, 7·29; N, 15·81; P, 0·954; S, 1·188 and Ca, 1·337. The question whether the protein itself is to be regarded as the fibrin-enzyme or whether its action on the formation of fibrin is due to an admixture with another substance is left undecided by Hammarsten (Ergeb. der Physiol., 1902, **1**, i, 339).

The optimal temperature for thrombin is 40°.

For the chemistry of the coagulation of blood see Morawitz, Hofm. Beitr., 1903, **4**, 381; 1904, **5**, 133; L. Loeb, Biochem. Zentralbl., 1907, **6**, 829, 889; Pekelharing, Biochem. Z., 1908, **11**, 1.

ENZYMES OF FERMENTATION

Fermentation is not a chemically definite conception; by it are understood those processes which are brought about by lower organisms and the extent of which is great compared with the mass of the organisms taking part.

From a chemical point of view, fermentation enzymes can be contrasted with the hydrolytic enzymes in so far as fermentation reactions consist of pure decompositions and take place without any other substance, such as water or oxygen, being taken up; the best-known example is the alcoholic fermentation of the hexoses which is effected by zymase. On the other hand,

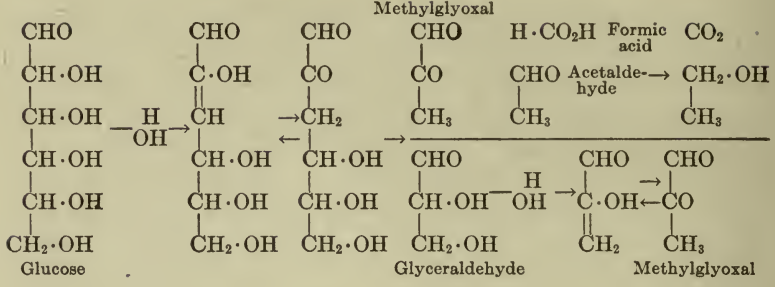
the enzyme of the acetic acid fermentation is regarded as belonging not to the same group but to the oxydases. The reactions underlying other fermentations have been investigated chemically only in very recent times and attention must be drawn especially to the work of Buchner and Meisenheimer; the existence of the enzymes which presumably take part in these fermentations has not yet been proved. The view that fermentations in general are to be referred to enzyme actions is a consequence of the discovery of E. Buchner, who, in 1897, succeeded in producing alcoholic fermentation in a pressed yeast juice free from cells and hence in showing that this fermentation is not dependent on the action of the living yeast.

Enzymes of Alcoholic Fermentation

The term zymase, in its wider sense, is used to indicate the sum-total of the enzymes which bring about the decomposition of certain of the hexoses in the sense of the following equation:

$$\underset{d\text{-Hexose.}}{C_6H_{12}O_6} = \underset{\text{Ethyl alcohol.}}{2C_2H_5\cdot OH} + 2CO_2.$$

As was assumed by Buchner and Meisenheimer and by Wohl, this chemical change is to be regarded as taking place in several separate stages. It was formerly thought that glucose gives rise to lactic acid under the action of an enzyme to which the name zymase was then applied in a more restricted sense. Buchner and Meisenheimer have, indeed, detected the formation of small quantities of lactic acid during fermentation, but Slator (Journ. Chem. Soc., 1906, **89**, 128) has shown that lactic acid, which is fermented with extreme slowness, can be only a bye-product and not an intermediate product of fermentation. It is now assumed that compounds allied to lactic acid are formed at an intermediate stage of the fermentation, which possibly passes through the following stages:



There is also uncertainty concerning the occurrence of methylglyoxal, which cannot be fermented by pressed yeast juice. Boysen-Jensen (Bot. Ber., 1908, **26**, 666; also Dissertation, Copenhagen, 1910) supposes the intermediate product to be dihydroxyacetone, an isomeride of glyceraldehyde, but it cannot be said that this has been proved to be the case; this assumption is, however, rendered probable by the fact that dihydroxyacetone is fermented readily and glyceraldehyde only slowly.

It is worthy of note that the transformation of glucose into alcohol+carbon dioxide can be effected by purely chemical means, the various reactions requiring, however, different catalysts:

Glucose→lactic acid (alkali as catalyst).

Lactic acid→acetaldehyde+formic acid (sulphuric acid as catalyst).

Acetaldehyde+formic acid→alcohol+carbon dioxide (rhodium as catalyst).

(Buchner, Meisenheimer and Schade, Chem. Ber., 1906, **39**, 4217; Schade, Zeitschr. f. physikal. Chem., 1906, **57**, 1.) Further, the author's investigations (Arkiv för Kemi, 1911, **4**, No. 8) show that, in ultra-violet light, lactic acid undergoes decomposition into alcohol and carbon dioxide.

According to a new and interesting investigation by Franzen and Steppuhn (Chem. Ber., 1911, **44**, 2915), formic acid is fermented as well as formed by living yeast and must hence be taken into account as an intermediate product.

As substrate for fermentation, mannose, galactose, or fructose may be used instead of glucose. Glucose and fructose exhibit no difference in their velocity of decomposition, and in the case of mannose there is only a slight deviation. Galactose, on the

other hand, is fermented only by certain species of yeast, including bottom fermentation beer-yeasts, and by their pressed juices; the fermentation takes place far more slowly than that of glucose (E. Fischer and Thierfelder, Chem. Ber., 1894, **27**, 2031; E. F. Armstrong, Proc. Roy. Soc., *B*, 1905, **76**, 600; Slator, Journ. Chem. Soc., 1908, **93**, 217). As, however, has been shown by Slator and by Harden and Norris (Proc. Roy. Soc., 1910, **82**, 645), the capacity of yeasts for fermenting galactose can be increased by cultivating them in solutions containing this sugar. Mention must also be made of Slator's view that the different hexoses are attacked by different enzymes: glucose and fructose by gluco-zymase, mannose by manno-zymase and galactose by galacto-zymase.

The mechanism of alcoholic fermentation is considerably less simple than was formerly supposed, a number of enzymes and subsidiary substances taking part in the formation of alcohol and carbon dioxide.

The first separation of zymase into a " zymase in a restricted sense " and a lactacidase must be given up, since the formation of lactic acid as an intermediate product has been shown to be improbable. And a special enzyme has now to be assumed for each of the changes indicated in the above scheme of reactions.

Of great importance for the elucidation of the nature of fermentation is Harden and Young's discovery of the co-enzyme of zymase. By filtration through a film of gelatine under a pressure of 50 atmospheres, pressed yeast juice can be divided into a filtrate and a residue, which are separately inactive towards sugar but produce fermentation when again mixed (Harden and Young, Proceedings of the Physiol. Soc., Nov. 12, 1904, see Journ. of Physiol., 1904, **32**, i; Proc. Roy. Soc., *B*, 1906, **77**, 405).

The substance in the dialysate resists boiling (thermostable) and undergoes hydrolytic decomposition and hence destruction by enzymes (lipases) of the yeast juice.

On the other hand, the zymase itself—which does not traverse the gelatine filter—is destroyed when heated and is presumably a protein substance, being attacked by the proteinases or proteases of the yeast juice. From this attack it is protected by a thermostable substance—antiprotease (Buchner and

Haehn, Biochem. Z., 1910, **26**, 171). This antiprotease is, like the co-enzyme, destroyed by lipase, but is more stable than the co-enzyme towards hydrolytic agents and towards heat.

Both enzyme and co-enzyme are precipitated from the yeast juice by acetone, but the latter less readily than the former, so that a certain degree of separation can be attained by fractional precipitation (Buchner and Duchaček, Biochem. Z., 1909, **15**, 221).

Alcoholic fermentation by means of pressed yeast juice is facilitated by the addition of a phosphate. Harden and Young (Proc. Roy. Soc., *B*, 1906, **77**, 405) have made the important discovery that, during the period of enhanced fermentation, the amount of carbon dioxide produced exceeds that which would have been formed in the absence of phosphate by a quantity exactly equivalent to the phosphate added—$CO_2 : R_2'HPO_4$.

These two investigators consider that the phosphate reacts with the hexose in the yeast juice in the following manner:

$$(1)\quad 2C_6H_{12}O_6+2R_2HPO_4=2CO_2+2C_2H_6O \\ +2H_2O+C_6H_{10}O_4(PO_4R_2)_2.$$

The result is an ester of hexosediphosphoric acid, the salts of which have been more closely investigated by Young (Biochem. Z., 1911, **32**, 177).

During the fermentation, the hexosediphosphate accumulates in the solution, but as soon as fermentation ceases, this ester undergoes hydrolysis in the yeast juice, thus.

$$(2)\quad C_6H_{10}O_4(PO_4R_2)_2+2H_2O=C_6H_{12}O_6+2R_2HPO_4.$$

This hydrolysis is effected by a "hexosephosphatase." These reactions will be considered further in Chapter VII.

Occurrence. Zymases do not only occur in yeasts, but are extraordinarily widespread throughout the whole of the animal and vegetable kingdoms. There is now scarcely room for doubt that the combustion of sugar in the animal organism and also in higher plants begins with decompositions completely analogous to those brought about by yeast. Deviation from these occurs only in the final phase of the reaction, since in living animal organs and living plants alcohol is formed only when oxygen is lacking. On the other hand, the intramolecular respiration of sugar is absolutely identical with alcoholic fermentation.

This view, as far as the higher plants are concerned, is due especially to E. Godlewski, Palladin and Kostytschew. Also the change known as glycolysis, occurring in the animal body, must be closely allied to fermentation.

Animal glycolytic enzymes or zymases have been prepared especially from the blood, spleen, pancreatic tissues and muscle.

Mme. N. Sieber (H., 1903, **39**, 484; 1905, **44**, 500) has obtained three glycolytic enzymes in the form of stable powders from blood-fibrin and spleen:

(a) soluble in water,

(b) soluble in neutral salt solutions, and

(c) soluble in water or alcohol (peroxydase).

All these enzymes contain nitrogen and give the reactions of the proteins. Their ash contains iron, manganese and phosphoric acid, but not copper. The second of them gave the following mean composition: C, 52%; H, 7·5% and N, 15%.

Tincture of guaiacum is turned blue directly by enzymes (a) and (b), but only in presence of hydrogen peroxide by (c). Röhmann and Spitzer's reagent (a dilute alkaline solution of α-naphthol and paraphenylenediamine) is coloured by all three enzymes in absence of hydrogen peroxide. It is doubtful if these oxydase- or peroxydase-reactions are related in any way to the ability to bring about the combustion of sugar. In any case the essential constituents of the enzyme-complexes obtained by Mme. Sieber are fermentation-enzymes and not oxidation enzymes.

Further the glycolytic enzyme found by Cohnheim (H., 1903, **39**, 336; 1904, **42**, 401) in muscle, from which he extracted it in an inactive state, is not oxydasic in character. It is a decided endo-enzyme like yeast-zymase and is obtained from the frozen, subdivided muscle by pressing or by extraction with an ice-cold, isotonic solution of sodium oxalate; the oxalic acid must then be precipitated with the calculated quantity of calcium chloride (H., 1906, **47**, 253). Corresponding with this enzyme, there exists a co-enzyme or activator which, like that of yeast-zymase, is thermostable. Cohnheim prepared it from the pancreatic tissue of the cat.

The objections raised by Claus and Embden (Hofm. Beitr., 1906, **6**, 214 and 343) to the investigations of Cohnheim have been refuted by the latter author. Reference

must be made to Stoklasa's work (Chem. Ber., 1905, **38**, 664; Arch. f. Hygiene, 1904, **50**, 165) which likewise indicates the existence of a zymase in the muscles and also in the milk.

It is, however, remarkable that such a capable experimenter as A. Harden, working in conjunction with H. Maclean (Journ. of Physiol., 1911, **42**, 64), was unable to detect alcoholic fermentation by animal tissues (liver, kidneys, pancreas, flesh, etc.) or by juices or powders prepared from them.

That the intramolecular respiration of plants is to be regarded as a zymase fermentation has already been mentioned (Palladin and Kostytschew, H., 1906, **48**, 214; Kostytschew, Bot. Ber., 1908, **26**, 167). Also by means of seedlings of Hordeum distichum, Pisum sativum and Lupinus luteus, Stoklasa, Ernest and Chocenský (H., 1907, **50**, 303; 1907, **51**, 156) have clearly shown the action of the enzymes of intramolecular respiration. The arguments opposed to Stoklasa's earlier experiments can scarcely be advanced against the work just referred to.

Palladin (Bot. Ber., 1905, **23**, 240) holds the view that the carbon dioxide respired by plants arises in three ways: 1. By the enzymes combined with the protoplasm; Palladin calls this portion, nucleo-carbon dioxide and the corresponding enzymes, "carbonases." 2. By the protoplasm itself (apparently directly) under the influence of various irritants—irritant-carbon dioxide. 3. By the action of oxydases. In another place (Zeitschr. f. physikal. Chem., 1909, **69**, 187, Arrhenius-Festschrift), the author has indicated that he is unable to agree entirely with Palladin's views, especially as regards carbonase and the rôle of oxydases and reductases in the respiration process. The opportunity must, however, not be neglected to direct attention to the many remarkable observations communicated by Palladin to the Berichte der deut. botan. Gesellschaft.

Preparation of yeast-juice. One kilo of washed and well-pressed bottom-yeast is mixed with 1 kilo of fine sand and 300 grms. of kieselguhr and is ground, in 4–6 lots, in a large mortar until the mass becomes soft and doughy. In this way a large proportion of the yeast-cells are broken. The dough is then enveloped in a press-cloth and pressed in a hydraulic press, the pressure being raised to about 90 kilos

per sq.cm. and maintained at this value for an hour. About 400 c.c. of a clear, pale-brown, viscous juice, containing only a very small number of living cells, are thus obtained (Eduard and Hans Buchner and M. Hahn, Die Zymasegärung, Munich, 1903, p. 58).

From the yeast-juice the zymase can then be precipitated with alcohol or acetone, which, however, throws down a large amount of proteins, carbohydrates and salts at the same time. In order to obtain very active preparations, the yeast-juice must be poured into a large excess of acetone (10 volumes); only in this way can the precipitate formed be obtained so free from water that the chemical changes occurring in it are reduced to a minimum (Buchner and Duchaček, Biochem. Z., 1908, **15**, 221).

The following simple method for obtaining zymase-preparations has been recently discovered by von Lebedew (C. R., 1911, **152**, 49; Bull. Soc. Chim., 1911, [iv], **9**, 744):

It consists in drying the yeast at a temperature of 25–30° and macerating with water for 2 hours at 35°; the filtered liquid exhibits considerable fermentative activity. According to the author's experience, Munich yeast (from Schröder's factory) and many other yeasts are suitable for this purpose, but this is not the case with all yeasts.

According to Rinckleben (Chem. Zeitung, 1911, **35**, 1149), zymase can also be obtained by plasmolysing fresh yeast with glycerol.

Preparation of Permanent Yeast. In addition to those described above, another method is known by which the fermentative activity of yeast-cells can be separated from the true life functions. When yeast is introduced into alcohol or acetone (Albert, Chem. Ber., 1900, **33**, 3775), the cells are killed without their fermenting power being destroyed. Well pressed yeast (500 grms.) is thoroughly disintegrated, placed on a hair-sieve and the whole dipped into a basin containing 3 litres of acetone. By alternately raising and lowering the sieve in the liquid, and by means of a small brush, the whole of the yeast-cells are passed through the sieve in 3 or 4 minutes. The yeast is then left in the acetone for 10 minutes, being frequently stirred meanwhile. Most of the liquid is next poured off and the yeast, after being pumped as dry as possible, is again

treated with acetone, filtered and drained. The residue is well kneaded with 250 c.c. of ether, filtered, dried in the air, finely ground and then dried for 24 hours at 45°.

This preparation has been placed on the market under the name of " zymin" by A. Schröder, of Munich.

The best antiseptics to use with yeast-juice are toluene and thymol.

Lactic Acid Bacteria-zymase

This is the enzyme by means of which lactic acid bacteria are enabled to decompose sugar into lactic acid. The enzymic nature of this transformation was demonstrated by Buchner and Meisenheimer (Chem. Ber., 1903, **36**, 635; Lieb. Ann., 1906, **349**, 125); they succeeded in obtaining an active permanent preparation of Bacillus Delbrückii which, however, only gave rise to small quantities of *dl*-lactic acid.

Preparation. The organism was cultivated at 40–45° in wort prepared from malt and rye.

The bacteria were subsequently separated by means of a centrifuge and dried on a porous tile. The mass was then introduced into 15–20 times its weight of acetone, with which it was ground for 10–15 minutes, the bacteria being pumped dry, washed with ether and dried in a vacuum.

OXYDASES

The action of oxydases is assumed in changes of very different kinds: oxidations of purine bases, conversion of alcohols and aldehydes into acids, transformation of simple and substituted phenols, amines, amino-acids, and derivatives of these compounds into quinone derivatives.

The following kinds are thus to be distinguished:

1. Purine-oxydases.
2. Alcoholases.
3. Aldehydases.
4. Phenolases.
5. Tyrosinase.

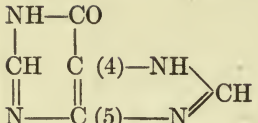
To these true oxydases must be added the peroxydases, which can hardly be separated sharply from the oxydases, and, especially with the latter, there remains still a good deal that is not clear, so that the division of this section can be considered only as a provisional one.

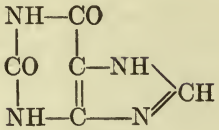
For the recognition of oxydases use has been made of a number of reactions, some of which are referred to below; they are not, however, given by all oxydases, which are, to a great extent, specific in their action.

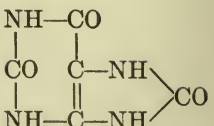
Blue coloration of guaiacum tincture or of α-guaiaconic acid; violet coloration of tetramethylparaphenylenediamine; brown coloration of *m*- and *p*-phenylenediamine alone or in presence of hydrogen peroxide (Aso); reddening of aniline acetate (C. R., 1896, **123**, 315) and blue coloration of α-naphthol (Bourquelot, C. R., 1896, **123**, 423; Soc. Biol., 1896, **46**, 896); reddening of aloïn (Schaer, Arch. der Pharm., 1900, **38**, 42; Kastle); oxidation of phenolphthalin to phenolphthalein (Kastle and Shedd, Amer. Chem. Journ., 1901, **26**, 526); oxidation of benzidine; leucorosolic acid (Kastle, Public Health and Marine Hospital Service of the U. S. Hygienic Lab. Bull., 1906, No. 26, 7–22), hydroquinone, pyrogallol, and guaiacol; oxidation of leuco-malachite green to malachite green (quantitative, spectro-photometric method of von Czyhlarz and von Fürth, Hofm. Beitr., 1907, **10**, 358); oxidation of aldehydes, e.g., salicylic aldehyde (Schmiedeberg, Arch. f. exp. Path., 1881, **14**, 288, 379), formaldehyde (Pohl; Arch. f. exp. Path., 1896, **38**, 65); oxidation of arsenious to arsenic acid; formation of diaminophenacin from *o*-phenylenediamine, and of indophenol from *p*-phenylenediamine and α-naphthol (Röhmann and Spitzer, Chem. Ber., 1895, **28**, 567).

The separation of iodine from potassium iodide has also been regarded as an oxydase reaction. But this action, as has been pointed out by Aso and more recently by Wolff and de Stoecklin, C. R., 1908, **146**, 1415), must be attributed to the nitrous acid occurring in plant juices.

As regards the oxidising enzymes of the purine bases, the action of xanthine-oxydase is best understood, owing especially to the work of Burian. This enzyme does not attack uric acid, which is so readily broken down by ordinary oxidising agents. Burian supposes it to be quite a general oxidising enzyme for (cyclic?) amidines which is unable to attack the double linking between the (4) and (5) carbon atoms of the purine nucleus:


Hypoxanthine


Xanthine


Uric acid

An enzyme which oxidises uric acid to allantoin was obtained by Wiechowski and Wiener (Hofm. Beitr., 1907, **9**, 232, 247, 295) from animal organs by treating the powdered organ (the cells being completely ruptured) with 0·05% soda solution.

The filtrates obtained after grinding with the soda solution are inactive, but if the emulsions are subjected to dialysis into 0·05% soda solution for 5–6 days, the enzyme of dog's liver passes completely, and that of ox-kidneys partially, into the filtrate. Enzymic liquids were also obtained by centrifugating the emulsions. Repeated precipitation and filtration yielded a protein-free preparation, which exhibited the total enzymic activity of the starting material.

Alcoholase: Oxydase of Acetic Bacteria.

This enzyme oxidises ethyl alcohol to acetic acid and thus catalyses a reaction for which energetic oxidising agents are otherwise necessary. The enzyme has not yet been separated from the bacteria, but its existence can be proved by killing the bacteria with acetone (Buchner and Meisenheimer, Chem. Ber., 1903, **36**, 637; Buchner and Gaunt, Lieb. Ann., 1906, **349**, 140).

Preparation. Large quantities of acetic bacteria (best Bacterium aceti, which can always be obtained by leaving beer in a glass dish exposed to the air) are cultivated on beer-wort to which 4% of alcohol and 1% of acetic acid have been added. As culture-vessels, shallow glass basins, as wide as possible, are most suitable; these are covered with a layer of cotton wool in order to prevent the entry of germs from the air. In the course of 4 or 5 days after inoculation, the bacteria form a fairly thick coating on the surface of the nutrient solution. The clear liquid underneath is syphoned off and the residue centrifuged to remove most of the liquid; the brittle bacterial membranes are then washed superficially with water and dried on porous tile. On the following day the

mass is introduced into 10–20 times its weight of acetone, rubbed to a fine powder and left for 10 minutes, after which it is filtered, washed with ether and dried in a vacuum over sulphuric acid. This procedure yields a yellowish powder which, however, only oxidises very small quantities of ethyl alcohol.

Aldehydases. Aldehydes, e.g., benzaldehyde and salicylic aldehyde, are oxidised by the extracts of many animal organs, but it is doubtful if this is an enzyme action. See the remarks of Bach (Chem. Ber., 1904, **37**, 3791) and the results of Dony-Hénault and van Duuren (Bull. Acad. roy. Belgique, 1907, 577).

Of the vegetable oxydases mention must first be made of the laccase from the lac-tree, which was discovered by Yoshida and studied in detail by Bertrand, who gives the following method for its preparation (Ann. de Chim. et de Phys., 1897, [vii], **12**, 115).

The thick sap of Rhus succedanea is mixed with 5–6 times its volume of alcohol, by which means the laccase is precipitated, whilst the phenolic derivatives which produce the blackening of the lac pass into the alcoholic solution. The solution is filtered through a cloth and the residue washed on the cloth several times with alcohol. It is then taken up in cold distilled water, only a small quantity of black substance remaining undissolved. The filtered solution is again precipitated with alcohol in large excess, the precipitate being collected on the filter and dried in a vacuum.

Laccase is obtained as a white substance having a neutral reaction and readily soluble in water. Bertrand regards it as a protein, although his preparation contained only 0·44% of nitrogen. He assumes its composition to be as follows:

Moisture (determined at 120°)	7·40%
Gum (arabans and galactans)	84·95
Laccase	2·50
Ash	5·17

The most essential constituent of the ash is manganese, which is present to the extent of 2·5%.

Laccase is extremely sensitive to acids (cf. Chapter III) and is destroyed by short boiling of its solution.

From other plants, such as Medicago sativa and Lolium perenne, Bertrand obtained preparations which he termed laccases, since, in neutral solution and in presence of manganese salts, they accelerate the oxidation of polyphenols. As the author has pointed out (H., 1909, **61**, 1), they are quite different from Rhus-laccase.

Preparation of Medicago-laccase. The fresh plants, at the commencement of the flowering stage, are chopped up and pressed. On standing, the juice obtained deposits dark flocks, from which it is separated by filtration. Alcohol is then added, the abundant precipitate thus formed being, to a large extent, taken up in water and again precipitated. This procedure is repeated thrice, the preparation obtained, after drying in a desiccator, being a white, dusty, highly hygroscopic powder, soluble in water with great readiness. According to Euler and Bolin, it consists mainly of the calcium salts of aliphatic hydroxy-acids. The separation of the mixture, effected by fractional crystallisation of the corresponding barium salts, shows it to contain glycollic, citric, malic, and mesoxalic acids. The oxidising action of these salts is described in Chapter IV.

Numerous attempts have been made to prepare laccases artificially. According to Bertrand, the oxidising agent of many plants is composed of manganese and a protein with a specific action. Trillat (C. R., 1904, **138**, 94, 274) assumed that the laccase regarded as a specific enzyme can be replaced by any protein or, at any rate, by certain classes of proteins; he has, however, no good foundation for this view.

Especially on the ground of his own experiments on Rhus-laccase, the author also regards as unsuccessful Dony-Hénault's attempts to attribute the action of laccase to the alkalinity of the preparations and thus to show that laccase-action is only an oxidation by means of manganese and hydroxyl-ions. The laccase preparations obtained by Bertrand's method are not alkaline, but are extremely active.

Further efforts to prepare artificial oxydases and peroxydases have been made by Martinand (C. R., 1909, **148**, 182), Wolff (C. R., 1908, **147**, 745), and de Stoecklin (C. R., 1908, **147**, 1489).

With other oxydases, so little has been done as regards purification and isolation that is has not been decided to what class of bodies these substances belong

Slowtzoff (H., 1900, **31**, 227) attempted to prepare the "laccase" of potatoes in a pure state, his purest preparation containing 12·8% N and 0·53% S.

Sarthou (J. de Pharm. et Chim., 1900, [vi], **11**, 482, 583; 1901, [vi], **13**, 464) obtained quite different numbers for his schinoxydase, namely, 6·28% N, 0·2% S, and 1·34% ash, from which he concluded the enzyme to be a nucleoprotein. Aso and Loew regard the oxydases as albumoses. But Rosenfeld's investigations (Dissertation, St. Petersburg, 1906) on the oxydase of the radish (Raphanus sativus) appear to indicate that this enzyme does not belong to the proteins. According to Rosenfeld the oxydase would be a crystalline substance, containing C, N, S, P, and K but not Fe or Mg.

Spitzer came to the conclusion that the oxydase of the liver is a nucleoprotein, but this enzyme, which effects the oxidation of salicylic aldehyde to salicylic acid, was further purified by Jacoby (H., 1900, **30**, 135). It is found to be soluble in water and non-diffusible, and to become inactive on heating, while it does not give the reactions characteristic of the proteins.

According to Tschirch and Stevens (Arch. der Pharm., 1905, **43**, 504), the oxydase of Japanese lac shows the pyrrole reaction. Bach and Chodat are of opinion that the oxydase consists of a peroxydase and an oxygenase, the former alone giving the pyrrole reaction (Bach, Chem. Ber., 1908, **41**, 226).

The oenoxydase of apples (Lindet, C. R., 1895, **120**, 370), pears, plums, grapes, and the fungus Botrytis cinerea, parasitic to grapes, must be a tannin-oxydase; by its action the flesh of the fruit is turned brown on exposure to the air.

Oxydases of doubtful individuality have been detected in numerous plants, e.g., Arum maculatum, olives ("olease"), barley and malt ("spermase"), coffee beans and yeast.

Should Battelli's observation (C. R., 1904, **138**, 651) on the oxidation of formic acid to carbonic acid by oxydases in presence of hydrogen peroxide be confirmed, an interesting organic oxidation will present itself. In this connection, mention may be made of Loevenhart's discovery (Chem. Ber., 1906, **39**, 130) that formic acid is oxidised to carbon dioxide by hydrogen peroxide in presence of iron, copper, etc.

A very remarkable oxidation, which is not yet understood, has been described by Zaleski and Reinhard (Biochem. Z., 1911, **33**, 449); it consists in the oxidation of oxalic acid in 1% solution to carbonic acid.

For information concerning other oxydases, see Battelli and Stern's résumé in Ergeb. der Physiol., 1912, **12**, 95.

Tyrosinase

(G. Bertrand, Bull. Soc. Chim., 1896, [iii], **15**, 791.) From tyrosine and its derivatives this enzyme forms, by oxidation, melanins—dark-coloured substances of unknown chemical composition.

Interesting data concerning the chemical aspect of the action of tyrosinase have recently been obtained by Abderhalden and Guggenheim (H., 1908, **54**, 331). They were able to show that ozone and tyrosine alone do not yield melanins, but that these are synthesised by oxidation from tyrosine or polypeptides containing it, on the one hand, and from phenols or amino-acids on the other. Similar colorations are obtained if the oxidation is effected with potassium dichromate instead of tyrosinase, and it is hence probable that tyrosinase contains an oxidising agent, the action of which is exerted along with that of the amino-acids of the plant-juice.

Tyrosinase attacks not only *l*-tyrosine itself, but also *dl*-tyrosine, tyrosine anhydride (Bertrand and Rosenblatt; Chodat), and a large number of polypeptides containing tyrosine. Suprarenin (adrenaline) is likewise oxidised, and all the cresols, resorcinol, *m*-toluidine, *o*-, *m*- and *p*-xylenols, thymol, carvacrol and naphthol; also, according to Bertrand, phenol.

The opinion expressed by Gonnermann that the specific action of tyrosinase consists of a hydrolysis, which is then followed by an oxidation (not specific), has been combated by Chodat and Bach.

According to Chodat, Zahorski and Freederickz (Arch. Sci. phys. nat., 1909, **27**, 306), the specificity of tyrosinase is conditioned by the presence of an activator which is stable to heat.

For the preparation of the enzyme, the disintegrated fungus is extracted with water and the extract precipitated with alcohol.

Occurrence. In numerous fungi of the species Boletus, Russula, Lactarius, Coprinus, Paxillus, etc. Also

in Merulius lacrimans, beet-juice, dahlia bulbs, potato skins, and Vicia faba. Tyrosinase is often accompanied by laccases.

From sepia, C. Neuberg has extracted an enzyme which resembles tyrosinase and acts on adrenaline.

PEROXYDASES

Under this name are included those substances which activate peroxides. Their typical reaction is the transference of oxygen from hydrogen peroxide to guaiaconic acid or to polyphenols.

Bach and Chodat (Chem. Ber., 1903, **36**, 600) have given a method for the preparation of peroxydase from pumpkins and horse-radish roots. Bach and Tscherniak have recently (Chem. Ber., 1908, **41**, 2345) obtained a peroxydase in the following manner:

Thirty kilos of turnips were pounded up and pressed and the juice obtained (20 litres) mixed with 2 litres of 96% alcohol in order to coagulate the gummy matters. After filtration, the alcoholic juice was precipitated by 130 litres of strong alcohol, the precipitate being filtered off, washed with alcohol and freed from precipitant in a vacuum. The crude peroxydase thus obtained (52 grms.) was kneaded with 600 c.c. of water, only a small portion of the substance passing into solution. The undissolved residue was filtered off and washed with a little water, and to 600 c.c. of the filtrate, containing only about 7 grms. of dry matter, 40 grms. of powdered basic lead acetate were added; the precipitate was pumped off and the clear filtrate (600 c.c.) treated with powdered sodium carbonate (21 grms.) until no further turbidity was produced. The alkaline filtrate was dialysed through parchment into distilled water. After 13 days the dialysate (670 c.c.) was mixed with 4·5 litres of 99% alcohol, and the precipitate thus formed collected, after 24 hours, on a hardened filter, washed with absolute alcohol and freed from the latter in a vacuum.

This preparation contained 7·87% of water, 81·66% of organic matter and 1·47% of ash; the percentage of nitrogen, calculated on ash-free material, was 3·44.

The peroxydase prepared by E. de Stoecklin in Chodat's laboratory from Cochlearia armoracia

contained 11·41% of water, 65·88% of organic matter and 22·71% of ash; the nitrogen-content was 3·43%. The activating power of this peroxydase was only one-tenth part of that of the above-mentioned preparation of Bach. Neither de Stoecklin nor Chodat (Schweiz. Woch. Chem. u. Pharm., 1905, **43**) obtained protein reactions with their peroxydases.

The author's experience (H., 1909, **61**, 1) indicates that dialysis is the best known method of purifying peroxydase preparations. From horse-radish Euler and Bolin obtained a preparation which increased continuously in activity when subjected to dialysis. The preparation showing the greatest activity per unit of weight contained 10·4% N and 2·5% of ash. Dialysis is rendered far more effective if the enzymic juice is previously treated with kaolin or other suitable adsorption agent in order to remove the proteins. Deleano (Biochem. Z., 1909, **19**, 266) proposes the use of colloidal ferric hydroxide for this purpose. Bach (Chem. Ber., 1910, **43**, 362) suggests the preliminary removal of the gummy matters by means of magnesium sulphate.

During recent years it has been repeatedly pointed out that the action of peroxydases can be obtained partly by purely inorganic materials (see Wolff, C. R., 1908, **146**, 781 and Martinand, C. R., 1909, **148**, 182), and partly by synthetic organic preparations (de Stoecklin, C. R., 1908, **147**, 1489).

As Moitessier, Lesser, von Fürth and von Czyhlarz, and Bertrand and Rogozinski (C. R., 1911, **152**, 148) have shown, the well-known guaiacum-blue reaction of the blood depends not on an enzyme, but on the hæmoglobin; it appears with undiminished intensity after boiling. Oxyhæmoglobin acts not only as a peroxydase but as an oxydase as well (de Stoecklin). Further, the oxidation phenomena in milk cannot depend on the presence of a peroxydase.

Thermolabile peroxydases do, however, exist and these must for the present be classed as enzymes.

CATALASES

(O. Loew, Rep. U. S. Dept. of Agric., 1901, No. 68.) After the ability to decompose hydrogen peroxide had been long regarded as a general property of the enzymes, O. Loew demonstrated the existence of special catalases. The discoverer distinguished two catalases occurring in plants, viz., an α-catalase which is not extractable by water and was regarded as a nucleoprotein, and a β-catalase soluble in water which was regarded as an albumose.

The chemical action of the catalases is analogous to that of the colloidal metals (Bredig), molecular (inert) oxygen being formed, together with water. Ethyl hydroperoxide is not decomposed (Bach and Chodat, Chem. Ber., 1903, **36**, 1757).

Occurrence: Extremely widespread in the animal and vegetable kingdoms. In blood (Senter; Lesser, Zeitschr. f. Biol., 1906, **48**, 1); in numerous organs (Kastle and Loevenhart; Liebermann; Battelli, C. R., 1904, **138**, 923); in milk (Raudnitz, Zeitschr. f. Biol., 1901, **42**, 91; Reiss, Zeitschr. klin. Med., 1905, **56**, 1; Faitelowitz, Dissertation, Heidelberg, 1904). Catalase is also found in virtually all plant-juices. Especially rich in this enzyme are many leaves, e.g., of clover, Rosa, Picea, which mainly contain the insoluble form of the enzyme; and certain seeds e.g., of the apple and peach, in which the enzyme occurs principally in the soluble form. Highly active catalases are obtained from fungi, e.g., Boletus scaber (Euler, Arkiv för Kemi, 1904, **1**, 357), and from the lower fungi, yeasts, and bacteria.

The preparation is usually carried out by precipitating the aqueous extracts with alcohol. Senter gives the following method of obtaining the catalase of the blood: Defibrinated ox-blood is mixed with 10 times its volume of carbonated water and left over night. It is then centrifuged and filtered and the liquid precipitated with an equal volume of alcohol, the alcoholic solution of hæmoglobin being poured off and the reddish-brown precipitate repeatedly washed with 50% alcohol. The precipitate is dried in a vacuum, ground to a fine powder, stirred with water and allowed to stand in ice for 2 or 3 days in order that the enzyme may be extracted completely. The solution is filtered through hardened filter-paper until it becomes quite

clear; the faintly yellow liquid thus obtained vigorously decomposes hydrogen peroxide with evolution of oxygen.

For the preparation of highly purified catalase-products, pig- or ox-fat is the most suitable starting material. The fat is disintegrated by means of sand and extracted with water at about 30°, the enzyme being then precipitated with alcohol and further purified by repeatedly dissolving in water and precipitating with alcohol (Euler, Arkiv för Kemi, 1904, **1**, 357; Bach, Chem. Ber., 1905, **38**, 1878).

In his first experiments (loc. cit.) the author obtained a preparation which still showed faint protein reactions and contained 14·5% N and 1·2% S but no phosphorus. A preparation made from defibrinated blood and similarly purified gave 14·1% N. A continuation (not yet published) of this investigation has yielded enzyme-preparations which are certainly of a higher degree of purity. The nitrogen-content diminishes as the purification proceeds, the final product, which exhibits considerable activity, containing 6·2% N and exhibiting Millon's and Molisch's reactions. Fat-catalase appears therefore to be not a protein, but mention must be made of the opposite results obtained by Bach (loc. cit.). The question of the protein character of catalase hence requires further investigation.

Reducing Enzymes (Reductases; Reducase)

A substance termed "philothion" was described by de Rey-Pailhade and was regarded by him and also by Pozzi-Escot as a reducing enzyme. With this view, however, the author is unable to agree, since the reactions described by de Rey-Pailhade and Pozzi-Escot are not enzymic in character. The existence of reducing enzymes has, indeed, not yet been demonstrated with absolute certainty. In most cases in which reduction has been observed, no attempt has been made to show that it is really due to an enzyme-action, i.e., a catalytic reaction, and is not merely a stoichiometric reduction by a readily oxidisable substance.

A substance which, at 70°, accelerates the reduction of methylene blue by formaldehyde, has been found to exist in milk (Schardinger). That this is a catalytic action has been

rendered probable by the investigations of S. Oppenheimer (Arb. a. d. Inst. f. exp. Therapie in Frankfurt, 1908, **4**) and of Trommsdorff (Centralbl. f. Bakt., 1909, **49**, 291). Schardinger's reaction is also hastened by colloidal platinum or iridium (Bredig and Sommer, Zeitschr. f. physikal. Chem., 1910, **70**, 34).

Bach has recently (Biochem. Z., 1911, **31**, 443) endeavoured to ascertain if Schardinger's enzyme, for which he proposes the name perhydridase, is related to the reducing enzymes of the liver and other tissues. He is of the opinion that the "reducase" of the liver is a mixture of enzymes and contains that of Schardinger. Further, the same reaction underlies the action of the systems: palladium—methylene blue—hypophosphate—water; palladium—methylene blue—aldehyde—water; milk-enzyme—methylene blue—aldehyde—water, this reaction consisting in decomposition of the water by the oxidisable substance with the help of a catalyst which forms a labile, strongly reducing compound with the hydrogen of the water.

In the researches of Abelous and his collaborators on horse-kidneys, bacterial action was not excluded. Indeed, as Abelous himself stated and the author has confirmed, no reduction takes place if the extract is filtered through a Chamberland candle (cf. the work of Heffter, Arch. f. exp. Path., Schmiedeberg-Festschrift, 1908, 29).

In the roots of plants and in seedlings, strongly reducing substances occur but these are not enzymic metabolic products (O. Schreiner and M. Sullivan). The same holds for reduction by micro-organisms. Kastle and Elvove (Amer. Chem. Journ., 1904, **31**, 606) have also given an interesting study on the reducing actions of plant-juices.

APPENDIX

In the above short account, mention has only been made of those enzymes, of the individuality and mode of action of which something definite is known. A large number of enzymes, which have received special names, have not been considered, as they do not differ essentially from the better-known representatives of the same groups.

But certain other enzymes, which have found no place in the

main enzyme groups, may be briefly referred to here, since their further study appears to be not without interest.

1. An isomerising enzyme, which converts mannose into glucose, is thought by Gatin (Soc. Biol., 1908, **65**, 903), to exist in the seeds of Borassus flabelliformis.

2. According to J. Parnas (Biochem. Z., 1910, **28**, 274) the liver contains a soluble enzyme which is able to accelerate Cannizzaro's aldehyde transformation, i.e., it converts aldehydes anaerobically into the corresponding alcohols and acids. Parnas suggests the name aldehydemutase, whilst Battelli and Stern (Biochem. Z., 1910, **29**, 130) apply the term aldehydase to a similar enzyme studied by them.

3. Kotake (H., 1908, **57**, 378) refers to an enzyme of ox-liver which demethylates caffeine, giving xanthine, hypoxanthine, *1*-methylxanthine, etc.

4. In the kidneys and liver, Gottlieb and Stangassinger (H., 1907, **52**, 1; 1908, **55**, 295, 322) found substances which convert creatine into creatinine and are apparently enzymic in character.

CHAPTER II

PHYSICAL PROPERTIES OF THE ENZYMES

Although the courses followed by most enzymic reactions can be represented by formulæ which hold for catalyses in homogeneous systems, and although also dynamics as yet affords little means of taking the state of solution or the colloidal condition of the enzymes into account, yet, in experiments with enzymic liquids, adsorption phenomena always make themselves more or less strongly felt and have, indeed, a determining influence on the general chemical behaviour of the enzymes.

Wherever a liquid is bounded by a vaporous space, there is formed at the surface a layer possessing properties different from those of the body of the liquid— this layer is termed the surface-layer. The latter has a tendency to diminish, and it is to this that the well-known capillary phenomena are due; on the interior of the liquid a pressure is exerted, termed the internal pressure.

The thickness of this surface-layer, which differs from the remainder of the liquid, is very small.

The pressure with which the surface-layer presses on the internal liquid is also very small, unless the relation between the surface of a substance and its volume—"the specific surface"—is very large, i.e., the distribution of the substance is considerable. The latter is especially the case with the so-called colloidal solutions.

We shall begin with a short theoretical consideration of surface phenomena, employing the method of representation given by Maxwell.

The wire *EF* (Fig. 1) is to be regarded as capable of moving freely along the rectangular wire *ABCD*. Within the frame *EBCF* is a layer of liquid which, in consequence of the surface-tension, tends to diminish and so draw the side *EF* upwards.

The weight is so chosen that it exactly compensates the pull of the liquid layer on the wire; increase of the load G would then result in rupture of the layer and decrease, in rise of EF.

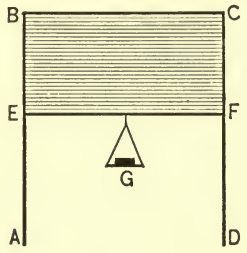


FIG. 1.

The pull of the layer is caused by its surface-tension, and, since both surfaces of the layer are active, corresponds with double the surface-tension.

If now the movable wire is displaced, by means of the weight G, from its highest position BC to the position EF, an amount of work has been done against the surface-tension which is proportional to G (made equal to the surface-tension) and to the magnitude of the surface produced.[1] This work evidently represents the surface-energy and is given by:

Surface-energy = Surface-tension × surface.

The surface-tension may also be expressed by the energy acting on unit-surface or by the force acting on unit-length, and is usually given in dynes per cm.

Owing to the small absolute values possessed by the known surface-tensions of liquids and aqueous solutions—about 100 $\frac{\text{dynes}}{\text{cm.}}$—the surface-energy of a liquid attains a considerable magnitude only when the surface is large, and hence becomes comparable with the other forms of energy of a substance only when the ratio of surface to volume—the "specific surface" (Wo. Ostwald)—exceeds a certain value, namely, about 10,000.

The physiologically most important and most interesting phenomena in this region are met with not in pure liquids, but partly in solutions and partly in "heterogeneous systems," in which a substance occurs in a very fine state of division and which Wilh. Ostwald has named disperse systems.

Solutions. It can be stated generally that the con-

[1] The surface-tension of liquids is independent of the expanse of the surface.

centration at the surface of a solution is different from that prevailing inside the solution. This can be demonstrated experimentally and also follows from thermodynamical considerations. The derivation given below follows that given by Millner [Phil. Mag., 1907, (6), **13**, 96] and was also deduced by H. Freundlich in his "Kapillarchemie."

Suppose n molecules to be dissolved in a certain volume v. The concentration has then not the uniform value $\frac{n}{v}$, but is greater (or smaller) in the surface-layer (or interior). If the excess per unit of surface ω is indicated by σ, the excess of concentration in the whole surface will be $\sigma\omega$ and the concentration in the interior of the liquid, $c = \frac{n - \sigma\omega}{v}$.

Consideration of a thermodynamic cycle leads to the differential equation:

$$\frac{d\tau}{dv} = -\frac{dp}{d\omega}, \quad \ldots\ldots\ldots \quad \text{(I)}$$

where τ is the surface-tension and p the osmotic pressure.

This expresses the fact that the surface-tension changes with the volume and, therefore, also with the concentration, if the osmotic pressure changes with the magnitude of the surface, and this can only happen if the concentration of the solution depends on the magnitude of the surface and is therefore different from the concentration in the latter.

If equation (I) is developed as a function of c, it gives rise to

$$\frac{d\tau}{dv} = \frac{d\tau}{dc} \cdot \frac{dc}{dv} = -\frac{c}{v} \cdot \frac{d\tau}{dc} \quad \ldots\ldots\ldots \quad \text{(II)}$$

and

$$\frac{dp}{d\omega} = \frac{dp}{dc} \cdot \frac{dc}{d\omega} = -\frac{\sigma}{v} \cdot \frac{dp}{dc}. \quad \ldots\ldots\ldots \quad \text{(III)}$$

The excess of concentration per unit of surface is hence given by

$$\frac{d\tau}{dc} = -\frac{\sigma}{c} \cdot \frac{dp}{dc}. \quad \ldots\ldots\ldots \quad \text{(IV)}$$

If the change of surface-tension with concentration is known, σ can be calculated from (IV). If the osmotic pressure obeys the simple gas-laws, then $\frac{dp}{dc} = RT$, and hence

$$\frac{d\tau}{dc} = -RT\frac{\sigma}{c} \quad \text{or} \quad \sigma = -\frac{c}{RT} \cdot \frac{d\tau}{dc}.$$

So far as the surface-tension of inorganic solutions has been measured, it exhibits a linear increase with the concentration, i.e., $\frac{d\tau}{dc}$ is positive and constant and therefore σ is negative and $\frac{\sigma}{c}$ constant. Hence we arrive at the result:

If the surface-tension of a solution diminishes with increasing concentration, the dissolved substance is more abundant in the surface; but if the surface-tension of a solution increases with augmented concentration, the concentration is less in the surface than in the interior.

The law is also expressed as follows:

A dissolved substance is adsorbed if it lowers the surface-tension; in the opposite case the adsorption will be "negative." It is, however, not advisable to designate as adsorption, the accumulation of dissolved substance at the surface of the solvent.

To the substances which lower the surface-tension of water belong most compounds which are not strong electrolytes, such as alcohols, glycerol, fatty acids, etc. When the surface-concentration attains a certain value owing to the tendency of these substances to collect at the surface of their aqueous solutions, the head of the osmotic pressure is held in equilibrium.

Unlike these substances, many salts increase the surface-tension of water towards air and, presumably, also towards other media, so that an increase of the concentration at the surface, like that just described, cannot then take place. On the other hand, it must be pointed out that Donnan and Barker (Proc. Roy. Soc., *A*, 1911, **85**, 557) have recently found, in certain cases, values for the surface-concentration (adsorption) which agree in order of magnitude with those calculated from Gibbs's equation.

What is understood by the surface-tension of a solution must be more strictly defined. Since the surface-tension depends on the concentration and since in every fresh surface a concentration is gradually attained which differs from that inside the liquid, it is evident that a newly-formed surface, which has not reached a condition of concentration-equilibrium with the interior of the solution, possesses a different surface-tension

from one already in stable equilibrium. The latter value is suitably termed the s t a t i c and the former the d y n a m i c surface-tension.

ADSORPTION

Of the possible cases of adsorption, those exhibited at the limiting surface between a solution and a solid body are of the greatest interest in this connection. Use is often made of such adsorptions in the study of enzymes.

These phenomena have, to some extent, been known for a long time, although the facts have only recently been satisfactorily collated.

Attempts have been made, especially during the last few years, to conceive of adsorption as a capillary phenomenon. But the above thermodynamical law showing the connection between the change of surface-tension and the adsorption has up to the present not proved very fertile. In the experimental proof it was necessary, except in one special case, to assume that the surface-tension of water-gaseous space proceeded parallel with that of water-adsorbent; and there was nothing to indicate that this was the case. A "negative adsorption" which should, according to the above theory, occur with electrolytes, has never been observed at the surface of separation between salt solutions and solid adsorbing material (H ä g g l u n d , H., 1910, **64**, 294).

Quite recently A r r h e n i u s (Medd. Nobel-Inst., 1911, **2**, 7) has subjected the experimental results of Miss F r a n c e s H o m f r a y , A. T i t o f f and G. C. S c h m i d t to calculation.

As regards the influence of the quantity of the adsorbing material, it is the m a g n i t u d e of its surface which is of the first importance; for one and the same preparation the quantity of substance adsorbed is, under similar conditions, proportional to the active surface. Of especial importance is the experimental result of the investigations of G. C. S c h m i d t (Zeitschr. f physikal. Chem., 1910, **74**, 689), namely, that the quantity adsorbed increases only to a maximum, no matter how high the concentration of the surrounding solution rises. This maximum is proportional to the amount of the adsorbing medium and varies with its nature.

G. C. Schmidt has investigated the simplest mathematical formulation of his results, namely,

$$\frac{dx}{dc}=k(s-x),$$

where s represents the maximum adsorption, x the quantity adsorbed, and c the concentration of the surrounding solution. But the integral equation derived from the above expression is not in agreement with the observations. On the other hand, the formula deduced theoretically by Arrhenius,

$$k\frac{dx}{dc}=\frac{(s-x)}{x},$$

holds generally for adsorption phenomena at low temperatures. This formula, on integration, gives

$$\log_{10}\frac{s}{s-x}-0{\cdot}4343\frac{x}{s}=\frac{1}{k}\cdot c.$$

Here x represents the amount condensed on 1 grm. of the adsorbing medium (charcoal), s the maximum value of this amount, c the osmotic pressure of the solute (or the pressure of the surrounding gas which is adsorbed), and k a constant.

Since the value of s is determined directly from the observations, the formula contains only one arbitrary constant, whilst that previously in general use, namely,

$$\frac{x}{m}=kc^n,$$

contains two, k and n. In spite of this, however, the new formula agrees much better with the observations.

Another empirical formula for the adsorption-equilibrium embracing a wider region was given by Freundlich (Zeitschr. f. physikal. Chem., 1907, **57**, 385):

$$\frac{v}{m}\log\frac{a}{a-x}=\lambda=\alpha\left(\frac{a}{v}\right)^{-\frac{1}{n}}.$$

In this expression a indicates the total quantity of solute and v the volume of the liquid. The magnitude λ is independent

of the quantity of adsorbing substance, but is a function of a and v, or, more strictly, of the ratio between them; α and n are magnitudes depending only on the temperature and on the nature of the solute.

The adsorption-equilibrium determined by the given formula must be completely reversible and independent of the path by which it is reached. The adsorbing medium charged with adsorbed substance must give up the latter to the pure solvent until a new equilibrium is attained.

This reversible adsorption is often followed by a consequent phenomenon—the fixing of the adsorbed substance—brought about partly by a change of this substance (which may become insoluble, for instance) and partly by a chemical reaction with the adsorbent—a reaction which, in many cases, leads to the destruction or denaturation of the adsorbed material. Many colouring matters are fixed from true solutions, but this more stable union takes place especially with colloids, in particular with proteins, toxines and enzymes. This fixation is non-reversible. With the toxines, the sum-total of the phenomena of antitoxine-formation is highly involved and has given rise to keen controversies. With enzymes, quite analogous processes are known, e.g., the combination of trypsin and antitrypsin, and the fixing of various enzymes by charcoal (S. G. Hedin, H., 1907, **50**, 497), to which reference will be made later.

It must here be mentioned that all adsorption phenomena are by no means to be attributed to one and the same cause.

Besides the mechanical adsorptions already mentioned, there exist a large number of adsorption phenomena caused by chemical transformations, and this is especially the case with acid and basic dyes, which are not adsorbed mechanically, but combined chemically, by animal fibres. L. Michaelis has repeatedly pointed out the chemical nature of many adsorption processes.

As Michaelis rightly stated (Oppenheimer's Handbuch, II, **1**, 390), of all known substances, charcoal and cellulose are the only ones with which mechanical adsorption occurs. "Almost all other substances known as adsorbents, such as silicic acid, kieselguhr, kaolin, arsenic sulphide, mastic, ferric hydroxide, clay and zirconium hydroxide, have practically

no mechanical adsorptive power and, in general, do not adsorb electro-indifferent substances like alcohol, acetone and sugars. They adsorb only substances which can occur in the form of ions or of electrically-charged suspensions, the adsorption taking place only in accordance with their electrical charge. Thus silicic acid adsorbs only such substances as migrate towards the cathode, and ferric hydroxide only those migrating to the anode." With these substances, then, it is always a question of a neutralisation between an acid and a basic compound at the surface of the adsorbing medium. According to Michaelis and Rona (Biochem. Z., 1908, **15**, 196), even adsorption by charcoal is not always purely mechanical, but sometimes takes place by means of electrical forces.

If two substances are adsorbed from a solution by an adsorption medium, they may replace one another (Michaelis). This fact is evidently closely related to G. C. Schmidt's discovery that the adsorption reaches a maximum. This phenomenon plays a part in the experiments of Hedin (H., 1909, **63**, 143), who found that the retardation of rennet-action by charcoal is counteracted by other substances, e.g., serum, white of egg, etc.

Colloids. The adsorption phenomena of greatest interest in the study of the enzymes are those in which colloids take part. On the one hand, these substances can, in the solid or gelatinised condition, function as adsorption media, and, on the other, they are themselves largely adsorbed by solid substances.

In the following considerations, we may limit ourselves to one of the two large groups into which colloids are divided, namely, the so-called emulsion-colloids or emulsoids, and may omit any description of the suspension-colloids or suspensoids which play virtually no part in enzymic solutions or in investigations of these.

While the suspensoids, to which, for example, colloidal metals belong, are classed with the true macroscopic suspensions, the emulsoids are so closely related to the crystalloids that no sharp limit can be drawn between them. With a number of classes of bodies, increase of molecular weight is accompanied by the appearance of a tendency to form complexes and thus pass into

the colloidal state. Good examples of this are presented by the condensation products of glucose—dextrin and starch—E. Fischer's polypeptides, and, according to F. Krafft's investigations (Chem. Ber., 1895, **28**, 2566; 1896, **29**, 1328) especially the fatty acids. Whilst the lower fatty acids exhibit normal ionisation and normal osmotic pressure, sodium laurate, $C_{12}H_{23}O_2Na$, for example, occurs principally in doubled molecules and sodium oleate, $C_{18}H_{33}O_2Na$, in 20% solution, produces no measurable elevation of the boiling point and thus behaves as a substance of infinitely large molecular weight (Krafft). The variation in properties is hence continuous from the emulsion-colloids to the crystalloids. The osmotic pressure and the magnitudes related to it are very small, even on the basis of the simplest possible molecular formula, and become still smaller with the increasing tendency to complex-formation accompanying increase of molecular weight. The values then often fall within those due to inefficient methods of purification or within the unavoidable limits of error, or else are of little significance owing to the material employed being chemically ill-defined. But where highly-condensed substances, such as starch, glycogen, etc., can be obtained in a state of considerable purity, the depressions of freezing point and elevations of boiling point indicate molecular weights of at least 100,000, these values being only minimal ones.

As regards the molecular weights of the enzymes—with the exception of the oxydases of Medicago which were investigated by the author and Bolin (H., 1909, **61**, 1)—nothing certain is as yet known. Great care should be exercised in drawing conclusions concerning the molecular magnitudes of enzymes from the diffusion experiments of R. O. Herzog and Kasarnowski (Zeitschr. f. Elektrochem., 1907, **13**, 527; Biochem. Z., 1908, **11**, 172) on commercial enzyme-preparations, partly on account of the very small enzyme-contents of these preparations and partly owing to the considerable weakening to which they must have been subjected during the investigations. With one of their purest invertase preparations, Euler and Kullberg (H., 1911, **73**, 335) have made diffusion experiments, the results being calculated by means of the author's formula, $D\sqrt{M}=\text{const.}$ (Wied. Ann., 1897, **63**, 273). For the coefficient of diffusion at 17° the value 0·037

was obtained and this gave—with considerable extrapolation, it is true—the molecular weight as 27,000. This number represents a minimal value and holds for neutral solution.

With substances of such great molecular magnitudes, Brownian movement begins to become visible. This movement is shown by small particles suspended or dissolved in a colloidal state in a solvent and consists of a continuous irregular motion, which increases in rapidity with the fineness of the suspended substance and with diminution of the internal friction of the liquid.

This motion is to be regarded as an expression of the general molecular motion of matter, as has been assumed by the kinetic theory of heat since the middle of last century. Brownian movement has been thoroughly studied in the case of suspension-colloids, but not with emulsion-colloids.

In the ultramicroscope of Siedentopf and Zsigmondy the emulsoids mostly show only a cone of diffused light, i.e., the particles are generally too small to be perceived ultramicroscopically as discrete forms.

Particles visible in the microscope have diameters down to about 2.10^{-5} cm. (microns). As submicrons are known those particles which are perceptible only in the ultramicroscope, whilst those the existence of which can only be perceived indirectly, even in the ultramicroscope, are termed amicrons and have diameters of from 5.10^{-7} to 1.10^{-7} cm.

Everything goes to indicate that colloidal solutions are to be regarded as mixtures of larger and smaller molecular aggregates, which are able to change into one another with greater or less rapidity. The most active chemically must always be the smallest and therefore the really dissolved parts, which explains why, in chemical transformations, colloidal substances follow the laws of reaction derived theoretically for dissolved substances.

The surface-tension of water is very considerably depressed by emulsion-colloids, as is shown by qualitative observation. According to Quincke (Wied. Ann., 1888, [iii], **35**, 580), the value of σ for a 10% tannic acid solution is 29% less than that for water; the surface-tension of a dilute gelatine solution is 28% less. Consequently these substances exhibit a marked

tendency to concentrate at the surfaces of the solutions and are strongly adsorbed.

As regards the first phenomenon, the accumulation of the colloid at the surface, this is exercised in an especially striking manner in the formation of solid membranes of peptone at the surface of gelatinous peptone, either with or without some chemical change.

Just as clearly is the concentration of emulsion-colloids at the surface of aqueous solutions seen if the liquid is shaken and the composition of the foam examined. Further, the great "head-retaining" properties of many solutions, e.g., of albumins, constitute an indication of accumulation of these substances.

SOLID, NEUTRAL ADSORPTION-MEDIA

Charcoal has often been used as an adsorbent for enzymes. Thus, S. G. Hedin (Bio-chemical Journ., 1906, **1**, 484; 1907, **2**, 81, 112; H., 1907, **50**, 497) showed that trypsin is adsorbed by animal charcoal; if a sufficient quantity of the latter is employed, the adsorption is complete. Also, at first it is reversible. But later the process which has been already mentioned and is not uncommon with adsorbed, organic substances, viz., fixing, takes place. Fixing is a relatively slow process and the amount of trypsin fixed increases with the amount of animal charcoal and with rise of temperature. Whilst, therefore, water is no longer able to extract the fixed trypsin from the charcoal, casein is able to do so and, the higher the temperature, the more completely is this the case. Charcoal and talc act in a similar manner towards rennet (Hedin, H., 1909, **60**, 364), which can also be removed from the charcoal by the substrate.

According to the same author, the α- and β-proteases occurring in ox-spleen are likewise taken up by animal charcoal in similar proportions.

In agreement with Hedin's results are those of E. Buchner and F. Klatte (Biochem. Z., 1908, **9**, 436), who found that trypsin is adsorbed from dilute solution by threads of silk, wool and cotton, strips of linen, paper and agar-agar and by asbestos and glass-wool.

Lipase can be completely removed from either an alkaline or an acid solution by means of charcoal or kaolin (L. Michaelis and P. Rona, Biochem. Z., 1907, **4**, 11; L. Michaelis, ibid., 1908, **7**, 488. See also L. Michaelis and M. Ehrenreich, ibid., 1908, **10**, 283).

Especially noteworthy is the fact that, in the adsorption of colloids by charcoal, etc., the colloids exert a reciprocal influence, and that even crystalloids, such as glucose, may diminish the capacity of charcoal to take up other crystalloids, presumably by altering the surface-tension.

On this fact depends the phenomenon, studied by Hedin (H., 1909, **63**, 143), namely, that the retardation of rennet-action produced by charcoal is prevented by various substances.

With these neutral adsorption media, which are generally employed as powders, must be classed those which adsorb when in the form of solid layers, such as cellulose as filter-paper (on this depend Gruss's investigations [Bot. Ber., 1908, **26a**, 191 and 1909, 27, 313] on the capillary analysis of enzymes), and the materials of the various filter-candles, e.g., the Chamberland-filter. Their behaviour towards enzymes is characterised principally by their ability or inability to retain the enzymes when solutions of the latter are filtered. Here, too, the molecular magnitude or the size of the particles of the substance to be filtered comes into play, the filter acting not only as an adsorption medium but directly as a sieve; with emulsion-colloids, however, the adsorption is usually the more important process. Of great interest are the investigations of Holderer (C. R., 1909, **149**, 1153; 1910, **150**, 230, 285 and 790), who showed that the permeability of the Chamberland-filter for enzymes depends on the concentration of the hydrogen ions in the solution. If the solution is neutral towards phenolphthalein ($OH' = 10^{-6}$), no adsorption takes place, the filter being permeable; but if the liquid is neutral to methyl orange, the filter-candle is impermeable. This is found to be the case with invertase, catalase, pepsin and emulsin. The following data show the behaviour of the enzymes towards the most common filtering materials.

Chamberland-filter

The following are retained:

Lipases of various origins (Fermi and Pernossi, Ann. Inst. Pasteur, 1889, **3**, 531).

Yeast-invertase (Fermi and Pernossi, ibid.).

Zymase (Buchner, Zymasegärung, Munich, 1903).

Pepsin and trypsin.

The following pass through:

Maltase (Croft Hill, Journ. Chem. Soc., 1898, **73**, 636).

Stomach-steapsin (Volhard, Zeitschr. f. klin. Med., 1901, **42**, 414).

Liver-aldehydase (Jacoby, H., 1900, **30**, 135).

Proteinases of malt (Fernbach and Hubert, C. R., 1900, **130**, 1783; **131**, 293).

Parchment

These are retained:

Pepsin (Hammarsten, Maly's Jahresber., 1874, **3**, 160).

Peroxydase (Euler and Bolin, H., 1909, **61**, 82.)

These pass through:

Invertin, amylase, and, slowly, emulsin, trypsin and pepsin (Chodschajew, Arch. de Physiol., 1898, 30, 241).

Rennet and pepsin pass through unstretched amnion-membrane (Jacoby, Biochem. Z., 1906, **1**, 53).

Rennet, invertin and catalase pass through intestinal membrane (Vandevelde, Biochem. Z., 1906, **1**, 408).

Rennet, invertin and catalase do not pass through cellulose walls (thimbles from Leune, Paris) (Vandevelde, loc. cit.).

Collodion-membranes

The following is retained:

Pepsin (Strada, Ann. Inst. Pasteur, 1908, **22**, 982).

The following pass through:

Emulsin and lactase (Bierry and Schaeffer, Soc. Biol., 1907, **62**, 723).

Trypsin partially: after activation with kinase, completely (Strada, loc. cit.).

Since colloids in the sol condition do not, in general, diffuse through animal membranes and colloidal skins, very different membranes may be employed as colloid-filters.

In the gradation of the colloid-content, the permeability of a filter may be varied at will by employing a substratum of cellulose, etc., so that the solution may be fractionally filtered. On this principle Bechhold constructed his ultra-filter, in which, as colloids, acetic collodion and gelatine are especially used (Zeitschr. f. physikal. Chem., 1907, **60**, 257; 1908, **64**, 328).

Fibrin flocks have proved effectual for the adsorption of many enzymes, such as rennet, pepsin (Jacoby, Biochem. Z., 1907, **4**, 21) and trypsin (Buchner and Klatte, Biochem. Z., 1908, **9**, 436). Numerous other coagulated proteins also exhibit marked adsorptive properties. (See also Bayliss, Adsorption in its relation to enzyme action, Kolloid. Zeitschr., 1908, **3**, 224.)

Solid acid or basic constituents. The chief of these are, on the one hand, the hydroxides of ferric iron, aluminium and magnesium, and certain of the so-called colloid metals (Rona; Deleano, Biochem. Z., 1909, **19**, 266), and, on the other, silicic acid. In this case, the principal action is a chemical union and not mechanical adsorption; this is shown by the selective adsorption of these sols, acid substances being vigorously adsorbed by the metallic hydroxides and basic ones by silicic acid; the same regularity appears in the reciprocal coagulation of the colloids.

Similar influences govern the adsorption of enzymes. As Michaelis found (Biochem. Z., 1907, **7**, 488), electronegative colloid solutions give with, say, invertin no precipitation, whilst the hydroxides of iron and aluminium completely adsorb this enzyme. Analogous behaviour is shown by pepsin (Biochem. Z., 1908, **10**, 283). On the other hand, the adsorption of amylase and saliva-diastase (ptyalin) depends on the reaction of the medium. Zymase appears to be a neutral substance (Michaelis and Rona) and is consequently not adsorbed by ferric hydroxide; but the co-enzyme of zymase seems to be readily adsorbed by ferric hydroxide (Resenscheck, Biochem. Z., 1908, **15**, 1).

To the acid adsorption media belongs also kaolin, which

hence adsorbs mainly basic substances. Since the rule, that acid and basic adsorption media adsorb respectively basic and acid substances, has proved generally valid, we are able, from observations on adsorption, to draw conclusions concerning the electro-chemical nature or the charge of adsorbable substances, in particular of the enzymes. These conclusions are remarkably well confirmed by other facts.

Electric transference. Under the influence of a difference of electric potential, emulsion-colloids migrate in the same manner as particles suspended in water; this effect is known as cataphoresis. But, whilst suspended particles migrate, as a rule, to the positive pole and themselves assume a negative charge (according to Coehn's rule, this behaviour depends on the fact that the dielectric constant of these particles is less than that of water), emulsion-colloids migrate partly to the positive and partly to the negative pole. This is comprehensible if it is borne in mind that emulsion-colloids have, as a rule, basic or acid properties, or—as amphoteric electrolytes —may exhibit the one or the other character according to the medium in which they exist. As with the ions, the charge which they assume on ionisation determines the direction of migration. Further, the velocity of migration is not appreciably different from those of dissolved ions; according to Whitney and Blake, for gelatine particles it has the value

$$25.10^{-5} \text{ cm./sec.}$$

for 1 volt/cm., the corresponding value for sodium ions being 43.10^{-5}. On the other hand, in the cataphoresis of emulsion-colloids there appear various marked disturbances, caused partly by the migration of the particles from the two electrodes and their precipitation in the medium as oppositely charged sols.

How great is the dependence of the electric transference of proteins on the reaction of the medium is best seen from Pauli's researches (Hofm. Beitr., 1906, **7**, 531). Albumin poor in electrolytes shows no motion under a pressure of 250 volts, whilst even in 0·01 N-hydrochloric acid it assumes an electro-positive, and in dilute alkali a negative character.

Pauli and Handovsky (Biochem. Z., 1909, **18**, 340) and also Michaelis have recently subjected these questions to a fresh and thorough investigation.

The most important results concerning the electric transference of enzymes are due to Michaelis. He found firstly (Biochem. Z., 1909, **16**, 81) that, independently of the reaction of the medium, invertin migrates distinctly to the anode and is hence decidedly acid in nature. Pepsin also exhibits a strong negative character as, in neutral and even in markedly acid solution, it migrates solely to the anode. Shortly afterwards (Biochem. Z., 1909, **17**, 831), it was also found possible, with a concentration of hydrochloric acid exceeding $\frac{1}{50}$N, to obtain electric transference of pepsin to the cathode, agreement with the adsorption analysis being therefore complete. On the other hand, trypsin and diastase behave also in the electric field as amphoteric substances, migrating to the anode or cathode according to the reaction of the solution; diastase is, however, more strongly positive and trypsin more strongly negative.

Michaelis (Biochem. Z., 1909, **19**, 181; 1910, **28**, 1) has determined the "relative acidity" of certain enzymes. If

$$K_a[\text{undissociated albumin}] = [H^+][\text{Alb}^-]$$

and

$$K_b[\text{undissociated albumin}] = [OH^-][\text{Alb}^+],$$

then, in an iso-electric state, i.e., when equal members of positive and negative albumin-ions are present,

$$\frac{K_a}{K_b} = \frac{[H^+]}{[OH^-]}.$$

This quotient assumes the following values:

Malt-amylase, 1	Trypsin, $10^5 - 10^8$
Serum-albumin, $10^2 - 10^3$	Pepsin, 5.10^9
	Yeast-invertase, ∞.

The relation between the "iso-electric constant," I, and the relative acidity, R, is expressed by the equation

$$I = \frac{R^2}{k_w},$$

where k_w is the dissociation constant of water.

For a very pure pepsin, Pekelharing and Ringer have recently (H., 1910, **75**, 282) established the iso-electric point.

Bierry, V. Henri and Schaeffer have also carried out experiments on the transference of enzymes—with dialysed enzyme solutions (Soc. Biol., 1907, **63**, 226; Biochem. Z., 1909, **16**, 473). The enzymes investigated were: amylases of animal and vegetable origin, invertin from yeast and from Helix pomatia, emulsin from almonds and from Helix pomatia, lactase from Helix pomatia, rennet (Hansen's) and catalase from the liver. Only one of these enzymes, namely, pancreas-amylase, migrated to the cathode, all the rest going to the anode.

Even in 0·01N-sodium chloride solution, albumin assumes an electro-positive character, whilst in dilute alkali it shows electro-negative behaviour. According to Pauli's original results, albumin poor in electrolytes shows no migration; but more detailed investigations by Pauli and Handovsky (Biochem. Z., 1909, **18**, 340) and, especially, by Michaelis (ibid., 1909, **19**, 181) show that neutral albumin or albumin at the iso-electric point—with an acidity of [H] = about 10^{-6}—does not remain stationary but migrates to both the anode and the cathode at the same time.

Literature: Michaelis, Dynamik der Oberflächen, Dresden, 1909.

The precipitating action of salts on the colloids has been studied in great detail, especially as regards the influence of alkali salts on the proteins.

The fundamental investigations on this subject and the application of precipitation with salts to the fractionation of mixtures of proteins are due to Hofmeister, who, as early as 1887, arranged the salts in the order of their precipitating actions. His work has been considerably extended in more recent times by Wo. Pauli (Hofm. Beitr., 1902, **3**, 225; 1903, **5**, 27; 1905, **6**, 233; 1906, **7**, 531; Biochem. Z., 1909, **17**, 235, etc.).

It has been found that the precipitating properties of the salts are composed additively of the actions of the cathions and anions. The following series begins with the ion showing the greatest precipitating, or the least dissolving power:

SO_4, HPO_4, CH_3CO_2, Cl, NO_3, Br, I, CNS

Li, Na, K, NH_4.

With the exception of LiCl, this series agrees perfectly with the one given below which was obtained by the author (Zeitschr. f. physikal. Chem., 1899, **31**, 360 and 1904, **49**, 303) for the salting-out of non-electrolytes; the table gives the mean, relative, molecular depression of solubility $(l_w - l_s) : l_s$, where l_w is the solubility of the non-electrolyte in water and l_s its solubility in a salt solution of normal concentration.

NH_4NO_3	0	KCl	0·23	$\frac{1}{2}ZnSO_4$	0·31
KI	0·02	$\frac{1}{2}BaCl_2$	0·24	$\frac{1}{2}K_2SO_4$	0·32
KBr	0·05	$\frac{1}{2}CaCl_2$	0·24	$\frac{1}{2}Na_2SO_4$	0·35
KNO_3	0·08	NaCl	0·25	$\frac{1}{2}Na_2CO_3$	0·36
$NaNO_3$	0·10	$\frac{1}{2}(NH_4)_2SO_4$	0·29	NaOH	0·36
LiCl	0·21	$\frac{1}{2}MgSO_4$	0·31		

It is therefore beyond doubt that the same phenomenon is being dealt with in the two cases. It will be especially seen that ammonium and magnesium sulphates, which are most frequently used for the precipitation of proteins and also of enzymes, are likewise active towards crystalloids.

The remarkable fact that the rapidity with which the electrolyte is added influences the completeness of the precipitation, has been subjected to detailed study by Freundlich and by Höber.

If small quantities of an acid or an alkali are added to the protein solution, the precipitating actions of the salts are modified and the order of the ions changed (Posternak, Ann. Inst. Pasteur, 1901, **15**, 85, 169, 570). The precipitation of proteins by the salts of the alkaline earth metals also exhibits peculiarities, which have been studied by Pauli (Hofm. Beitr., 1903, **5**, 27). Unlike the precipitations produced by alkali metals or magnesium, those effected by salts of the alkaline earths are irreversible; they differ however, distinctly from those brought about by heavy metals. Further, in strongly acid solutions, especially on addition of alkali salts, irreversible precipitations occur.

Especially marked is the effect of addition of acids on the action of neutral salts on protein When a little acid is added to carefully-dialysed serum-albumin, not only does the latter become capable of migrating to the negative pole, but its coagulability by heat and by alcohol is impaired. At the same time its viscosity undergoes considerable increase. Excess of acid, however, restores the precipitability by alcohol and lowers the

viscosity again. Addition of any neutral salt has the same effect as excess of acid in restoring the coagulability of acid-albumin by heat or alcohol and in diminishing the viscosity.

The emulsion-colloids are distinguished by the considerably greater internal friction of their solutions from the pseudo-solutions of the suspension-colloids, which often possess viscosities only slightly higher than that of the pure medium. Very small additions of salts or, more especially, of acids and bases, produce marked changes in the viscosity of proteins and the conclusion must be drawn that protein-ions give rise to greater internal friction than amphoteric protein.

The course of proteolysis has often been followed by measuring the viscosity without, however, the parallelism between the composition of the solution and its viscosity being sufficiently clearly proved.

Jellies. Many colloid-containing liquids which, to indicate their consistency, are termed sols, solidify on addition of salts, alcohol, etc., to an apparently homogeneous mass of peculiar semi-solid consistency—a so-called jelly. The best examples are silicic acid and alumina among the suspension-colloids and agar-agar and gelatine among the emulsion-colloids. The homogeneity is, however, only apparent. Although by no means in all cases, yet in many it can be seen under the microscope that the solidification is accompanied by a de-mixing. The course of this change has been followed microscopically by Hardy. But no sharp limit exists between sols and gels and Pauli was right when he emphasised the fact that all gradations exist between solid and liquid jellies, and that a jelly is nothing but a thick sol (Biochem. Z., 1909, **18**, 367).

According to Pauli (Hofm. Beitr., 1902, **2**, 1), salts or ions exert the same relative influence on precipitation as on gelatinisation, so that the series, SO_4—Cl—CNS, given above holds also for the melting- or solidifying-point of gelatine. A corresponding parallelism had formerly been observed by Hofmeister between precipitation and swelling.

CHAPTER III

ACTIVATORS (CO-ENZYMES), PARALYSORS AND POISONS

For the occurrence of enzyme reactions, activators or co-enzymes are undoubtedly of greater and more general importance than has been, until quite recently, supposed. In many cases, enzymic processes do not take place without the help of specific co-enzymes, which, indeed, always exert a considerable influence on the course of the reaction; so that those chemical substances which effect the activation or inactivation of the enzymes must be studied qualitatively and quantitatively in as complete a manner as possible. Sörensen and also Hudson have recently emphasised the marked influence of acids and bases, or of the concentrations of H^+ or OH^-, on enzyme action, and, by very complete investigations on invertase, catalase and pepsin, have obtained numerical expression of this influence. As regards the less general but none the less important influence of neutral salts and non-electrolytes, no such comprehensive study has been made, so that a résumé of the numerous qualitative data must suffice.

The term co-enzyme has been applied to a number of substances which take part in enzymic reactions. But this name, which has come into very general use, is not quite suitable, since it characterises the substances as enzymes, whilst it refers partly to inorganic and partly to organic, thermostable bodies of known compositions. It is therefore best to use the term "activator" for all substances which, specifically or otherwise, participate with an enzyme in the acceleration of a reaction.

Following the ordinary conception and method of nomenclature a distinction must be drawn between those bodies which convert "zymogens" into the active state—kinases—and those which, on the other hand, intensify enzyme actions. It may be, however,

as will be mentioned later, that kinases do not act in an essentially different manner from other activators.

KINASES OF UNKNOWN COMPOSITION

As is well known, tryptase, as it occurs in the pancreatic juice, is activated by a constituent of the intestinal liquid—enterokinase [1] (Pawlow and collaborators, 1900). Although enterokinase is regarded by various investigators, especially Bayliss and Starling (Journ. of Physiol., 1904, **32**, 129) and Zunz, as an enzyme, Hamburger and Hekma (J. de Physiol. et Pathol. gén., 1902 and 1904) have adduced important evidence in support of the view that the formation of trypsin does not consist in a new enzyme action on trypsinogen, but that a definite quantity of enterokinase can activate only a certain amount of pepsinogen.

The preparation of pure enterokinase was attempted by Delezenne (Soc. Biol., 1901, **53**; 1902, **54**). It can be precipitated from intestinal juice by calcium phosphate or alcohol. Also flocculent fibrin and red blood-corpuscles adsorb the kinase, the former quantitatively. Unlike most activators, enterokinase is not stable to heat, as, according to Hamburger and Hekma (loc. cit.), it is destroyed at 67° in less than 3 hours; but Bierry and Henri (Soc. Biol., 1902, **54**, 667) assert that it retains its activity after being heated to 120° for 20 minutes.

Enterokinase dissolves in 90% alcohol (Cohnheim, Arch. Sci. Biol., St. Petersburg, 1904, **9**, Suppl., 112).

Kinases which activate trypsinogen were found by Hongardy (Arch. internat. de Physiol., 1906, **3**, 360) in milk and by Delezenne in leucocytes, bacteria and fungi and also in fibrin (Soc. Biol., 1903, **55**, 27 and 132).

According to Morawitz (Hofm. Beitr., 1903, **4**, 381; 1904, **5**, 133) and others (for the literature, see Buckmaster, Science Progress, 1907, **2**, 51), a kinase of unknown character plays a part in the formation of thrombin; it converts thrombogen into α-prothrombin, which, in its turn, is changed into thrombin by lime.

[1] For its preparation, see Bayliss, Journ. of Physiol., 1904, **30**, 80.

Thrombokinase is not stable to heat. According to investigations by E. W. A. Walker (Proceedings of the Physiol. Soc., Dec. 16, 1905, see Journ. of Physiol., 1905, **33**, xxi) coagulated oxalate-plasma, which has been heated at 50° for 2 hours, coagulates not on addition of calcium chloride alone, but when blood or fresh tissue-extract is added at the same time. So that a thrombogen would be stable at 50°, whilst thrombokinase is destroyed at this temperature.

Walker found that saliva-amylase which had been inactivated by heating at 50-55° could be re-activated by blood and he therefore regarded this as the mutual action of a thermostable enzymogen and a kinase sensitive to heat.

Further investigation of these phenomena is to be desired.

In most other cases e.g., in that of the esterases in which the existence of "kinases" has been assumed from the results of experiments with artificially inactivated enzymes, it is the action of thermostable activators which has been observed.

That Rosenheim succeeded in separating the lipase of the pancreas from an activator by filtration, has been already mentioned (p. 10).

The composition of the organic activator stimulin, mentioned by Danilewski and more closely examined by Schapirow, is also unknown.

Cohnheim has recently found that an extract can be obtained from the boiled pancreas of the cat which strongly activates the glycolytic enzyme of the muscles. Nothing is, however, known concerning the chemical nature of this interesting activator. It is precipitated by phosphotungstic acid (Hall, Amer. Journ. of Physiol., 1907, **18**, 283).

SPECIAL ORGANIC ACTIVATORS

In the case of pancreas-lipase, the chemical nature of an organic activator has recently been established. According to the observations of Nencki, Pawlow and Bruno, Rachford, Magnus, and Loevenhart (Journ. of Biol. Chem., 1907, **2**, 391), bile intensifies the hydrolysis of fats by pancreatic juice; still greater effects were found by Donath (Hofm. Beitr., 1907, **10**, 390) to be produced by salts of the bile acids. These salts are contained in the co-enzyme,

resistant to boiling, obtained by R. Magnus (H., 1902, **42**, 149) from the liver. Lecithin has no action (Kalaboukoff and Terroine, Soc. Biol., 1907, **63**, 617) or only a slight one (Loevenhart and Souder, Journ. of Biol. Chem., 1907, **2**, 415). A similar intensifying action is produced by the sodium salts of the synthetic glycocholic and taurocholic acids. The co-enzyme can be separated from liver-lipase by dialysis. It must be emphasised that these substances accelerate the action of pancreas-lipase specifically and have no action on the lipase of the stomach (Laqueur, Hofm. Beitr., 1906, **8**, 281) or on that of the intestines (Boldyreff, Zentralbl. f. Physiol., 1904, **18**, 460; H., 1907, **50**, 394).

Of some importance is the observation of O. Rosenheim and Shaw-Mackenzie (Journ. of Physiol., 1910, **40**) that substances which exert a hæmolytic action, such as alcohol, soaps, saponins, digitoxin, increase the action of pancreas-lipase. Such activation is annulled by cholesterol. The hydrolytic action of pancreas-lipase is augmented also by blood-serum.

The action of the amylases is also intensified by bile salts (Wohlgemuth, Biochem. Z., 1909, **21**, 447). The action of these salts on the amylase of the pancreas is, as was pointed out by Buglia (Biochem. Z., 1910, **25**, 239), independent of the concentration of the enzyme.

That bile contains also an activator for trypsin has been long known (Rachford, Journ. of Physiol., 1899, **25**, 165; Delezenne, Soc. Biol., 1902, **54**, 283; von Fürth and Schütz, Hofm. Beitr., 1906, **9**, 28; Wohlgemuth, Biochem. Z., 1906, **2**, 264).

It is, however, doubtful whether bile salts represent a specific kinase, as it may be that they influence the condition of solution of the substrate. Mention must be made of Donath's view—that bile salts do not activate the ready-formed lipase, but accelerate the conversion of the lipase-zymogen into the enzymic state (Hofm. Beitr., 1907, **10**, 390).

Amino-acids are noteworthy as activators of amylase (see Chapter IV; also Ford and Guthrie, Journ. Chem. Soc., 1906, **89**, 76; Journ. Inst. Brewing, 1908, **14**, 61). Pancreatic juice is also activated by amino-acids. Wohlge-

muth (Biochem. Z., 1906, **2**, 264) observed this effect with glycocoll, alanine and other representatives of this group.

According to Reichel and Spiro (Hofm. Beitr., 1905, **7**, 504), lecithin accelerates the action of rennet. Of much greater importance is the activation to which zymase is subjected under the influence of lecithin and other organic compounds of phosphorus. The investigations of Harden, Young, and Buchner and Meisenheimer have shown that these phosphorus compounds constitute the active constituent in boiled pressed yeast-juice (compare p. 54 and Chapter IV).

ACIDS, BASES AND NEUTRAL SALTS

Between the actions of purely specific activators or catalysts and the general actions of acids and bases a definite limit can hardly be drawn. Thus, according to Hoyer's investigations (H., 1906, **50**, 414), the action of lactic acid on the lipase of the castor-oil seed can also be produced by other acids, the action being, however, greater than corresponds with the degree of dissociation of this specific "seed-acid." Similar relations are observed with pepsin.

Apart from the activation of the zymogens, acids and bases can influence enzymic reactions in two different ways, which must be clearly distinguished: firstly, the velocity of the reaction is changed and reaches a well-defined maximum for a certain concentration of the hydrogen-ions; secondly, acids and bases influence the stability or the decomposition of the enzyme itself, the stability also exhibiting a maximum for a certain concentration of the H^+ or OH^- ions.

Pepsin requires the presence of a free acid as an absolutely necessary activator. Pepsin solutions are, indeed, proteolytically active only when they contain positive pepsin-ions. In the organism it is the hydrochloric acid which converts the pepsin-forming secretion of the mucous membrane of the stomach—Langley's "pepsinogen" or pro-pepsin—into the active enzyme.

Numerous investigations have been made on the replacemene of the hydrochloric acid; of the older ones, those of Pflei-

derer (Pflüg. Arch., 1897, **66**, 605), von Moraczewski, Hahn (Virch. Arch., 1894, **137**, 597) and Sjöquist (Skand. Arch. f. Physiol., 1895, **5**, 277) may be mentioned. The following tables, from a paper by Larin (Biochem. Zentralbl., 1905, **1**, 484), and from that of Sjöquist, give the acids arranged in the order of magnitude of their accelerating action.

Larin		Sjöquist
1. Hydrochloric acid	7. Lactic acid	1. Hydrochloric acid
2. Oxalic acid	8. Formic acid	2. Phosphoric acid
3. Nitric acid	9. Malic acid	3. Sulphuric acid
4. Sulphuric acid	10. Acetic acid	4. Lactic acid
5. Tartaric acid	11. Butyric acid	
6. Citric acid	12. Valeric acid	

The relative actions of the acids are also dependent on the nature of the digested protein (Berg and Gries, Journ. of Biol. Chem., 1907, **2**, 489).

Attempts have naturally often been made to connect the accelerating actions of the acids with their strengths (affinity constants or degrees of dissociation). To the complicated nature of the reaction is due the fact that, between these two magnitudes no quantitative relation, but at most an approximate correspondence, has been found. A large part of the acid must form salts with the digesting protein and it appears that it is just these salts which are the cause of peptic decomposition. The hydrolysis of the protein salt, i.e., the hydrochloride, must diminish and the concentration of the salt for a given quantity of protein increase as the strength of the acid present increases. Since the hydrolysis is governed by the general condition of equilibrium

$$\underset{\text{protein}}{\gamma_2 C_2} \times \underset{\text{acid}}{\gamma_3 C_3} = \underset{\text{salt}}{\gamma_1 C_1} \times \underset{\text{water}}{\gamma_4 C_4}$$

where

γ_2 and C_2 are the degree of dissociation and the concentration of the protein
γ_3 " C_3 " " " " " acid
γ_1 " C_1 " " " " " salt
γ_4 " C_4 " " " " " water,

it is at once clear on what magnitudes the velocity of pepsin-digestion would depend and in what manner, if the concentration of the protein salt were the sole determining factor. But first of all account must be taken of the action of the acid on the enzyme

molecule, which is thereby converted from the inactive (zymogen) into the active condition, the acid presumably remaining combined with the enzyme during the whole course of the digestion.

Especially striking is the slight activity of sulphuric acid as shown in the above series; this is possibly to be attributed to the harmful influence which Grützner (Pfleiderer, Pflug. Arch., 1897, **66**, 605) found to be exerted by sulphates even in very small amounts. On the other hand, the very strong activating action of oxalic acid (Wroblewski and others) must be noticed. The position assigned by Sjöquist to phosphoric acid is possibly related to the activating influence often found to be exerted by phosphates.

Sörensen has made a thorough investigation of the influence of the concentration of the hydrogen ions on the velocity of digestion; this will be referred to in Chapter IV. It appears that the optimal action takes place in solutions the concentration of which with regard to hydrogen ions is about 0·06-normal.

The concentration of the free hydrochloric acid of the gastric juice was measured in 1889 by F. A. Hofmann (Zentralbl. f. klin. Med., 1891, **11**) by the physico-chemical inversion method. There are, however, objections to the use of this method for such measurements (compare Sörensen, Biochem. Z., 1909, **21**, 144).

As regards the quantitative determination of the influence of acids and bases, it is undoubtedly best to investigate the relation between the velocity of the reaction and the concentration of the hydrogen ions in the solution. As Sörensen has shown, the concentration of the hydrogen ions is most conveniently measured either electrometrically or colorimetrically by means of indicators.

Whether the activation of a zymogen by acids is a process differing from the acceleration of the action of an enzyme already in the active state has not yet been clearly established. In so far as the author's experiments go, no such difference exists and in what follows these two effects will not be treated separately.

Reynolds Green (Proc. Roy. Soc., 1890, **48**, 370) assumed that an acid is necessary for the activation of the lipase-zymogen of plants, and the subsequent lipolysis also depends on the presence of dilute acid.

From the results of his experiments on the influence of acids

on the splitting of fats by Ricinus-lipase, Hoyer (Chem. Ber., 1904, **37**, 1436) drew the conclusion that, for a given amount of fat or enzyme a definite, absolute quantity of acid is necessary for obtaining the optimal effect. Sulphuric, oxalic, formic, acetic and butyric acids are about equal in their capacity for initiating the enzyme action. Armstrong and Ormerod, whose experimental numbers are quoted in the next chapter, also found no connection between the activating actions of different acids and their dissociation constants. From the fact that, for a constant quantity of seeds, a definite minimal amount of acid is required for the maximum fat-splitting action, Hoyer concluded that the acid reacts chemically with the seeds during the decomposition of the fat.

Volhard (Zeitschr. f. klin. Med., 1901, **42**, 414 and **43**, 397) has carried out a series of interesting experiments on the sensitiveness of gastric lipase to acid and alkali. Gastric juice hydrolyses fat in both neutral and acid solutions, a concentration corresponding with 0·1-normal hydrochloric acid being required to diminish the action appreciably; on the other hand, the juice is extremely sensitive to minimal quantities of sodium hydroxide. The glycerine extract is, however, very sensitive towards hydrochloric acid and much more resistant to alkali. It may hence be concluded that the mucous membrane of the stomach contains a lipasogen which, in its behaviour towards acids and alkalis, differs from the stomach lipase (the so-called steapsin) itself.

Blood-lipase, investigated by Rona (Biochem. Z., 1911, **33**, 413), exhibits its optimal activity with a H^+—concentration of 1.10^{-7}—$0{\cdot}26.10^{-8}$, i.e., with an approximately neutral reaction.

According to Lintner and others, the action of malt diastase is accelerated only by excessively small quantities of weak acids. Ford finds that the optimal action occurs in neutral solution. Concentrations of acid as low as 0·001% of hydrochloric acid, cause retardation (Effront, Cole).

But with pancreas-diastase, rather higher concentrations of hydrogen ions—about 0·001-normal—are necessary for the activity to reach its maximum.

Saliva-amylase (ptyalin) was first examined in its relations to acidity and alkalinity by Hammarsten.

Numerous subsequent investigations have been made, with varying results (Chittenden, Langley). It is active in faintly alkaline and more so in neutral solution, while dialysed ptyalin acts on dialysed starch still better in an extremely faintly acid solution (Cole, Journ. of Physiol., 1903, **30**, 202, 281). But even 0·001% of hydrochloric acid exerts a retarding action. According to Foà's measurements, saliva is approximately neutral.

Inulinase also exhibits its optimal activity in a solution having a very slight acid reaction (0·0001–normal HCl). Even 1·5% soda solution completely destroys the enzyme (Bourquelot).

Concerning the optimal acidity of invertase we have very detailed information. Apart from the older work of O'Sullivan and Tompson and of Cole, two important series of experiments have recently been made by Hudson and by Sörensen.

The following curve (Fig. 2) is taken from Hudson's work; it refers to the temperature 32° and gives the total

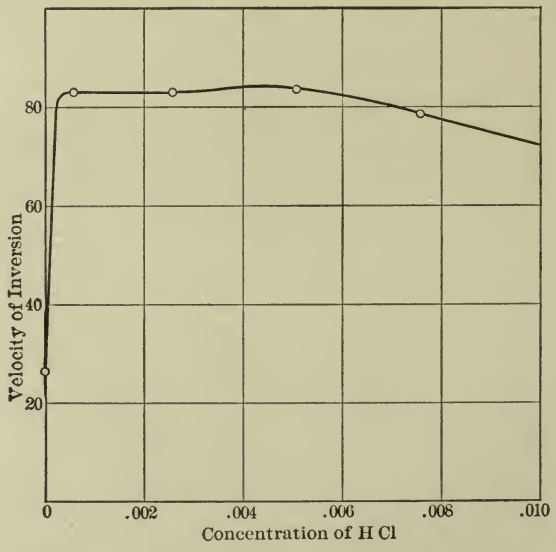


Fig. 2.

concentration of the hydrochloric acid in solution, i.e., the sum of the free and combined (with traces of protein) acid.

Hudson has proposed the following theory in explanation of the influence of acids and bases on the activity of invertase (Journ. Amer. Chem. Soc., 1910, **32**, 1220). Starting from the fact that invertase behaves as an amphoteric electrolyte and is hence capable of combining with both acids and bases, he regards the activity of the enzyme solution as proportional to the amount of enzyme which is not so combined. The values of the activity calculated in this way are found to correspond with the experimental numbers.

Sörensen (Biochem. Z., 1909, **21**, 144) found that the optimal proportion of sulphuric acid varies widely for different invertase solutions and, as would be expected, increases with the nitrogen-content of the enzyme solution; a similar relation would doubtless be found in the case of other strong acids. On the other hand, the optimal concentration of the free hydrogen ions was found to be the same to within 0·00003 ($p_H = 4{\cdot}4 - 4{\cdot}6$) in all the series of experiments, quite independently of the proportion of proteins, etc., in the invertase solution. The position of the optimum may be seen from Fig. 3. The three series of experiments were made with sulphuric acid at a temperature of 52°.

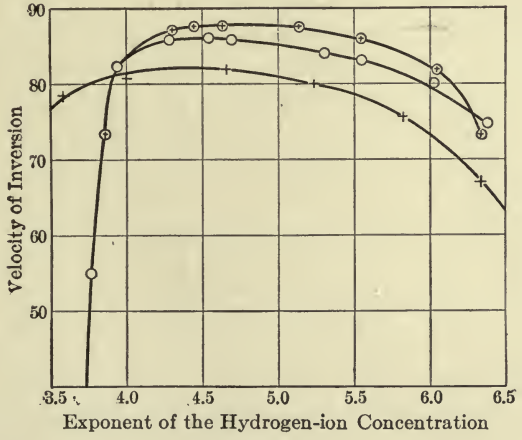


Fig. 3.

The concentration of the hydrogen ions influences also the course of the reaction, i.e., the constancy of the values of k

calculated from the formula: $k=\frac{1}{t}\log\frac{a}{a-x}$ (Sörensen); further reference will be made to this in Chapter IV.

As regards the influence of acidity on the destruction of the invertase, Euler and af Ugglas showed that at 50° the stability is a maximum when the concentration of the hydrogen ions is 10^{-6}. At lower temperatures this optimum is, naturally, not so well defined, as is shown by curves given by Hudson and Paine (Journ. Amer. Chem. Soc., 1910, **32**, 779). The numbers given by these investigators are as follows:

Concentration (grm.-mols. per litre) normal HCl.	Rate of destruction of invertase $k_2\times1000$	Concentration (grm.-mols.per litre).	Rate of destruction of invertase, $k_2\times1000$.
0·05	365	Distilled water	0
0·04	96	Normal NaOH	
0·03	42	0·01	3
0·02	4	0·02	11
0·015	1	0·03	50
0·01	0	0·04	245

The rate of destruction, k_2, was calculated from the activity A. The destruction follows the formula for unimolecular reactions, namely, $\frac{1}{t}\log\frac{A}{A-x}=k_2$, where A is the activity of the invertase at the beginning of the destruction and x is the activity after the destruction has proceeded for t minutes.

Lactase. According to Bierry and Salazar (C.R., 1904, **139**, 381) the optimal action takes place with an acidity of 0·001 N-HCl; 0·01 *N*-acid has a retarding action.

Pectinase (Bertrand's pectase) shows no action at all in 0·1% hydrochloric acid solution.

Among the proteolytic enzymes there are some—especially from plants—which, as already mentioned, show their optimal activity in a slightly acid solution. This is the case, according to Weis (H., 1900, **31**, 79) and Lintner (Zeitschr. f. d. gesamt. Brauwesen, 1902, **25**, 365), with the enzyme of malt, and, according to Vines (Annals of Bot., 1897, **11**, 563; 1898, **12**, 545; 1901, **15**, 563; 1902, **16**, 1; 1903, **17**, 237, 597; 1904, **18**, 289) with numerous plant-extracts containing ereptase and with yeast-extract. The autolysis of the substance of germinating plants also proceeds best in acid solution (Butkewitsch).

Emulsin shows its optimal action in neutral solution, as Auld has recently shown by exact experiments (Journ. Chem. Soc., 1908, **93**, 1251). For the decomposition of salicin by emulsin, Vulquin and Martini (Soc. Biol., 1911, **70**, 763) give the optimal concentration of hydrogen ions as $0 \cdot 36.10^{-4}$—$0 \cdot 41.10^{-4}$.

Papain, according to older statements, acts best in neutral solution; it is weakened by alkali, the activity being restored by hydrochloric acid (H., 1907, **51**, 488).

Similarly, the animal body contains, in addition to peptase, proteolytic enzymes which exhibit their action in acid solution. Among these are the proteolytic enzymes of the lymphatic glands, kidneys and spleen which were discovered by Hedin and Rowland (H., 1901, **32**, 341) and are retarded by alkali.

Numerous researches on the autolytic enzymes are in agreement in indicating that autolysis takes place only in acid solution or, at any rate, that it proceeds much more rapidly in acid than in faintly alkaline solution (Schwiening, Virch. Arch., 1894, **136**, 444; Hildebrandt, Hofm. Beitr., 1904, **5**, 463; von Drjewezki, Biochem. Z., 1906, **1**, 299). A detailed investigation of the influence of acids and alkalis on autolysis is due to Hedin.

Within certain limits of concentration, boric and salicylic acids cause increase of the autolysis of the liver over that occurring in chloroform-water. With the optimal concentration, the following proportions of the total nitrogen pass into solution (Yoshimoto, H., 1909, **58**, 341).

In chloroform-water	21·6%
In 1% boric acid solution	40·8%
In half-saturated salicylic acid solution	47·4%

Similar results were obtained by Kikkoji (H., 1909, **63**, 109).

According to Sachs (H., 1905, **46**, 337) the nucleases act best in faintly acid solution.

Pancreatic and intestinal juices, which act preferably with a neutral or alkaline solution, are not quite so sensitive to acids as the gastric juice is to alkalis. In aqueous solution pure tryptase is rendered inactive by a 0·01–*N* concentration of hydrogen ions. But presence of the substrate

diminishes the sensitiveness and 0·02% of lactic acid even accelerates tryptic digestion (Lindberger). Hence tryptase and ereptase hydrolyse proteins not only in alkaline or neutral solutions, but in certain cases also in slightly acid solutions. But it is found that even free carbonic acid retards the digestion. With trypsin, the origin of the enzyme also causes irregularities in this respect; such irregularities have led Pollak (Hofm. Beitr., 1904, **6**, 95) to assume the existence of a separate enzyme, glutinase.

Just as the optimal acidity is given by the concentration of the hydrogen ions in solution, so the optimal alkalinity is given by the concentration of the free hydroxyl ions. From the results of Dietze (Dissertation, Leipzig, 1900), Kanitz (H., 1902, **37**, 75) has calculated that tryptic digestion is most rapid when the solution has a concentration $\frac{1}{70}-\frac{1}{200}$-normal with respect to hydroxyl ions.

These values, which were obtained for fibrin-digestion, cannot, however, be applied immediately to all trypsin actions.

According to Kudo (Biochem. Z., 1909, **15**, 473), tryptase-digestion is retarded by an alkali-concentration of 0·0118% or 0·003-normal. The reversible retarding action is accompanied by an irreversible action, which destroys the tryptase.

In experiments on the velocity of decomposition of dipeptides by pancreatin and by ereptase, the author (H., 1907, **57**, 213) obtained very low results. Ereptase from pig-intestine showed a marked alkalinity-optimum, as is seen from the following figures obtained with glycylglycine:

0·1 N-glycylglycine. 5 grms. powdered ereptase per 100 c.c.

Concentration of alkali	0	0·04	0·05	0·075	0·10
Reaction constant, $k\times1000$	$<0·05$	7·0	6·2	2·6	0·2

With erepsin from germinating peas, the optimum was not so sharp (Arkiv för Kemi, 1907, **2**, No. 39).

Concentration of alkali	0	0·025	0·05	0·10
Reaction constant, $k\times10000$	0·7	10·3	9·0	5·7

When allowance is made for the salt-formation between the alkali and the glycylglycine, the dissociation constant of which as an acid amounts to $1·8\times10^{-8}$, the first of these tables gives

as the optimal concentration of the hydroxyl ions, 0·00002–normal. (Combination of the erepsin with alkali is here disregarded.)

Abderhalden and Koelker (H., 1908, **54**, 363) have carried out similar experiments, some of the results of which (series B, p. 380) are given below:

	(*a*) 1·5 mol. NaOH (calculated on the amount of dipeptide taken)	(*b*) 1·0 mol. NaOH	(*c*) Without NaOH, as control.
	4·0 c.c. of a $\frac{3}{32}$ mol. solution of glycyl-*l*-tyrosine.	4·0 c.c. of a $\frac{3}{32}$ mol. solution of glycyl-*l*-tyrosine.	4·0 c.c. of a $\frac{3}{32}$ mol. solution of glycyl-*l*-tyrosine.
	0·6 c.c. pancreatic juice.	0·6 c.c. pancreatic juice.	0·6 c.c. pancreatic juice
	0·07 c.c. intestinal juice	0·07 c.c. intestinal juice	0·07 c.c. intestinal juice
	0·55 c.c. N-NaOH	0·37 c.c. N-NaOH	1·6 c.c. water
	1·05 c.c. water	1·23 c.c. water	
Time in Minutes.	Rotations.		
6	+0·80°	+0·73°	+0·59°
15	+0·81°	+0·75°	+0·57°
41	+0·80°	+0·64°	+0·48°
174	+0·76°	+0·60°	+0·40°
260	+0·73°	+0·54°	+0·38°
378	+0·58°	+0·43°	+0·23°
1428	+0·49°	+0·31°	+0·09°

These results show that even small quantities of alkali retard the hydrolysis of glycyl-*l*-tyrosine by pancreatic juice+intestinal juice.

A number of investigations on the influence of alkalinity on the decomposition of proteins and peptones by erepsin have been carried out by Vernon (Journ. of Physiol., 1903, **30**, 330; 1904, **32**, 33). He distinguishes two ereptases—pancreatic and intestinal—both of which are accelerated by 0·4–1·2% of sodium carbonate, the intestinal ereptase being at the same time irreversibly destroyed. The erepsins of different animals exhibit varying sensitiveness towards alkali; the protective action of the proteins, studied by Vernon (cf. p. 115) may here be the determining factor.

Also the investigations of Bayliss (Arch. Sci. Biol. St. Petersburg, 1904, **11**, 261, Supplement) on casein indicate a smaller optimal alkali-concentration than Dietze's experiments with fibrin.

We have as yet no clear conception concerning the mode of action of the alkali in tryptic digestion. During the reaction

free alkali must disappear, since carboxyl groups are rendered free by the division of the protein-molecule into amino-acids. It might then be expected that this diminution of the hydroxyl ions would be rendered apparent in the course of the reaction. The obvious step is to test the applicability of one of the formulæ holding for negative auto-catalyses—processes in which the catalyst is used up by the reaction itself. It is found, however, that such formulæ do not correspond with the experimental numbers; on the contrary, in the experiments of Taylor and Bayliss, the coefficient k of the theoretical formula

$$k = \frac{1}{t} ln \frac{a}{a-x}$$

remains comparatively constant. With the help of a gas chain, T. B. Robertson and Schmidt (Journ. of Biol. Chem., 1908, **5**, 31) have recently studied the law according to which the hydroxyl ions diminish in concentration during the digestion. They find that this diminution may be expressed by a unimolecular formula if the OH-concentration is greater than 10^{-6} and by a bimolecular formula if this concentration lies between 10^{-6} and 10^{-7}. Definite conclusions in regard to the part played by the OH-ions in the digestion cannot be drawn from these results.

Hexosephosphatese, the enzyme that effects the esterification of the hexoses with phosphoric acid acts only in neutral or alkaline solution (Euler and Kullberg, H., 1911, **74**, 15).

Chymase (chymosin) is converted from the state of zymogen into the active condition by acids (Hammarsten, 1872) which, according to their efficiency in this respect, are arranged in the following order (equimolecular proportions): HCl, HNO_3, H_2SO_4, lactic, acetic, H_3PO_4 (Lörcher, Pflüg. Arch., 1898, **69**, 183). After the rennet is activated, it functions in either neutral or alkaline solution. The differences between rennets of various origins have been clearly indicated by a series of investigations by Gerber (C. R., 1907–1910, **145–150**; see references on p. 48).

The action of vegetable rennases, which at all temperatures clot raw milk less easily than boiled, is retarded by small quantities of alkali and accelerated by larger quantities of acid. Those which act on fresh milk with difficulty only at high temperatures are retarded by

acids having a higher basicity than two and also by quite small proportions of dibasic acids, larger proportions of which have an accelerating action; all other acids exert an intensifying effect. But those chymosins which clot raw milk more readily than boiled, are accelerated in their action by all acids.

With certain (calciphile) vegetable rennets, e.g., that from the sap of Maclura aurantiaca, the clotting of both raw and boiled milk is accelerated. In presence of basiphile rennet, the clotting of raw milk is only slightly hastened by small doses of HCl and is considerably retarded by medium amounts of the acid; the action on boiled milk is in all cases accelerated, but to a much less extent than with the really calciphile rennases, such as chymosin from calf's stomach.

The concentration of the H'-ions in milk has recently been measured electrometrically by van Dam (H., 1908, **58**, 295) and found to be $0{\cdot}14$–$0{\cdot}32 \times 10^{-6}$; the results obtained indicate that the time of clotting is inversely proportional to this concentration.

Parachymase (parachymosin) seems to be much more resistant to acids but much more easily destroyed by alkali, than chymosin (Bang, Pflüg. Arch., 1900, **79**, 425).

Sera showing slight thrombin action are activated by either acids or alkalis (Arch. f. klin. Med., **79** and **80**).

Zymase-fermentation is accelerated by small quantities of alkali (Buchner and Rapp, Chem. Ber., 1897, **30**, 2668) and retarded by slight amounts of acid.

The remarkable sensitiveness of laccase to acid, which was determined quantitatively by Bertrand, is shown by the numbers given in the next chapter.

Peroxydases are also paralysed by acids, of which larger quantities are required than in the case of laccase. The paralysing effect of acids is approximately proportional to their degree of dissociation (Bertrand and Rozenband, C. R., 1909, **148**, 297).

The influence of acids and alkalis on blood-catalases of various origins was first studied in detail by Jacobson (H., 1892, **16**, 340). The author has compared the behaviour of the catalases from fat and from Boletus scaber (Hofm. Beitr., 1905, **7**, 1).

Senter (Zeitschr. f. physikal. Chem., 1903, **44**, 257) found that acids cause considerable retardation of the action of catalase, without injuring the enzyme permanently. The length of the incubation period—the time during which the enzyme is

in contact with the acid before the hydrogen peroxide is added—has no substantial influence on the reaction. That very low concentrations of acid have a large effect is shown by the following figures:

Concentration of acid	Velocity constant
1/10,000-normal HCl	0·0011
1/20,000-normal HCl	0·0075
1/∞-normal HCl	0·0100

With hydrochloric and nitric acids the retarding action, according to Senter, varies very nearly proportionally with the third power of the acid-concentration. But Faitelowitz states that the "poisoning" of milk-catalase by hydrochloric acid is approximately proportional to the concentration of the acid.

In connection with the action of acids and bases, mention must be made of that of acid salts, of which the acid phosphates, carbonates, citrates, etc., have more especially to be considered. In this case, also, the hydrogen ions usually constitute the active component. Acid salts, or mixtures of neutral salts with the corresponding (weak) acids, maintain the concentration of the hydrogen ions in enzyme reactions constant within fairly narrow limits, since in their presence small quantities of acids or bases can only cause a slight change in the ionic equilibrium of the solution. Such acid salts or mixtures of salts are appropriately termed "buffers." As will be readily understood, amino-acids act in the same manner as acid salts, i.e., as buffers.

Activation by Salts. As has been pointed out by Delezenne and E. Zunz (Soc. Biol., 1906, **59**, 477 and **60**, 1070), calcium and magnesium salts exert a very marked activating and accelerating influence on tryptic digestion. [The harmful effect of calcium chloride, found by Malfitano (C.R., 1905, **141**, 912) with the protease of splenic fever is perhaps due to the optimum concentration of calcium being exceeded.]

It is assumed by Pawlow, by Bayliss and Starling (Journ. of Physiol., 1905, **32**, 129), and by Zunz that the transformation of pro-enzymes by kinases or by calcium salts represents a catalytic reaction. In support of this view E. Zunz has made the following experiments:

After inactive pancreatic juice had been left for 10–12 hours in an incubator with calcium chloride or nitrate and the calcium precipitated by ammonium oxalate, it was found to have a proteolytic action. Under the same conditions the juice was not activated in 1–2 hours. Analogous results were obtained with magnesium salts.

When added in the form of soluble salts, sodium, potassium, ammonium, zinc, beryllium, aluminium, cobalt, nickel, iron, manganese, uranium and copper had no effect. With salts of cæsium, rubidium and lithium, an activating influence was shown, but not regularly. For the numerous detailed results obtained by Zunz with regard to the activation of pancreatic juice, reference should be made to his complete monograph " Recherches sur l'activation du suc pancréatique " (Brussels, 1906–1907).

α-Pro-thrombase is converted by calcium salts into thrombase (Morawitz, Hofm. Beitr., 1903, **4**, 381; 1904, **5**, 133). Also pectinase, the vegetable clotting enzyme which transforms pectin into pectinic acid, functions only in presence of calcium (or barium or strontium) salts (Bertrand and Mallèvre, C.R., 1894, **119**, 1012; 1895, **120**, 110 and **121**, 726).

As, in addition to tryptase, ereptase (Abderhalden, Caemmerer and Pinkussohn, H., 1909, **59**, 293) and pancreatic lipase are intensified in their action by calcium chloride (Pottevin, C. R., 1903, **136**, 767; Kanitz, H., 1905, **46**, 482), it can no longer be asserted that calcium is a strictly specific activator. There is, however, no doubt that, in the cases named, calcium cannot be replaced by the alkali metals.

That the well-known discoveries of Jacques Loeb (Vorlesungen über die Dynamik der Lebenserscheinungen, Leipzig, 1906) on the specific action of calcium salts are related to these phenomena can scarcely be doubted.

On the other hand, purely chemical reactions are also known in which calcium acts as a specific catalyst. Thus, O. Loew (Chem. Ber., 1888, **21**, 270) found that lime is an especially suitable agent for the condensation of formaldehyde to sugar. The author has observed the same to be the case with the formation of formate (Chem. Ber., 1905, **38**, 2551).

A specific action, similar to that of calcium, appears to be exerted in certain cases by magnesium. The important part played by magnesium in plant life has been referred to by

Willstätter (Lieb. Ann., 1906, **350**, 48) in a very interesting paper.

Among the metals of biological importance as activators, manganese must also be numbered. As was first established by Bertrand, this metal is an essential constituent of the oxydases. It occurs in company with hydroxy-acids, with which, in the oxydases, it is combined. As manganese is known to be a powerful oxidising catalyst, its function in the oxydases is readily explained from a chemical standpoint.

The accelerating action of manganese on the decomposition of hydrogen peroxide by catalases is also related to these effects.

More remarkable is the fact that manganese salts hasten enzymic hydrolyses. Thus, as Hoyer (Chem. Zentralbl., 1905, II, 582) found, the splitting of fat by vegetable lipases is favoured by small quantities of manganese sulphate, whilst the diastatic enzymes of serum and of pancreatic juice were shown by A. Gigon and T. Rosenberg (Skand. Arch. f. Physiol., 1908, **20**, 423) to be activated energetically by the same salt, even in the concentration 0·001%.

According to Kayser and Marchand (C. R., 1907, **144**, 574, 714; 1907, **145**, 343; 1910, **151**, 816) and also Fernbach and Lanzenberg, glucose is fermented more quickly and completely in presence of manganese nitrate (0·1–0·5%) than without it.

Iron salts serve as general catalysts for purely chemical oxidations, and, just as they accelerate the decomposition of hydrogen peroxide by colloidal platinum, they intensify the action of the catalases, when used either alone or together with manganese salts. They also exert a specific accelerating influence on the action of the tyrosinases (Durham, Proc. Roy. Soc., 1904, **74**, 310; Bach, Chem. Ber., 1910, **43**, 364). Whether the undoubtedly important rôle of iron salts in the organism is played in conjunction with the enzymes, or whether it is preferably independent, cannot yet be decided.

According to Bach (Chem. Ber., 1910, **43**, 366), aluminium salts intensify the action of tyrosinase even more than manganese salts do. Less marked, but still appreciable, are the effects of calcium and magnesium salts.

It may here be mentioned that hydrogen peroxide increases

the action of the digestive enzymes (Vandevelde, Hofm. Beitr., 1904, **5**, 558).

Among the most noteworthy of inorganic activators are the alkali phosphates. Apart from the action of primary and secondary phosphates as "buffers", the phosphates exert a marked, specific influence on certain enzyme reactions. Mention must first of all be made of zymase-fermentation for which, as explained elsewhere, the presence of a phosphate is necessary. Further, ammonium and calcium monophosphates accelerate diastatic action (Effront), whilst for the action of ptyalin, phosphates are absolutely necessary (Roger, Soc. Biol., 1908, **65**, 374.) The same is the case with liver-diastase (see later). This effect possibly explains the following observation made by Roger (Soc. Biol., 1907, **62**, 833, 1021, 1070):

Human saliva is inactivated by heating for 10–15 minutes at 85–100°, but if a small quantity of fresh saliva is subsequently added, the mixture has a much greater saccharifying action than the added saliva alone. This observation has recently been extended and completed by Bang (Biochem. Z., 1911, **32**, 417).

As has been shown by Harden and Young, the phosphates play an extremely important part in fermentation. These actions are described in detail elsewhere (Chapters I and IV).

Rennetic action is also favoured by small quantities of monosodium phosphate (Gerber, Soc. Biol., 1908, **64**, 1176) possibly owing to the alteration effected in the acidity. The same cause may, perhaps, explain the activation of laccase by disodium phosphate in certain oxidations (J. Wolff, C. R., 1909, **149**, 467).

In connection with these intensifications by phosphoric acid, it may be mentioned that Chittenden observed an acceleration of peptic digestion by arsenious acid. According to Izar, autolysis is sometimes hastened by arsenic.

Also in their effect on alcoholic fermentation, arsenates and arsenites correspond to some extent with the phosphates, as is shown by recent important results obtained by Harden and Young and referred to at length in Chapter IV.

Alkali Salts. The salts of the alkalis are partly accelerating and partly inhibiting in their action.

The salt which has been most thoroughly investigated is sodium chloride. According to Osborne, Bierry and

Schäffer (Soc. Biol., 1907, **62**, 723), Cole (Journ. of Physiol., 1903, **30**, 202, 281), Wohlgemuth (Biochem. Z., 1908, **9**, 10), it facilitates the actions of diastase, maltase and ptyalin. But the majority of the enzymes, e.g., invertase, peptase, tryptase and the zymases and catalases (see, for instance, Lockemann, Thies and Wichern, H., 1909, **58**, 390) are retarded by sodium chloride.

Amylase is accelerated, often considerably, by small quantities of the chlorides, nitrates, sulphates, phosphates, vanadates and alums of the alkali metals (Lintner, Journ. prakt. Chem., 1887, [2], **36**, 481; Effront, C. R., 1892, **115**, 1324; Grüss). Ptyalin is slightly accelerated by potassium iodide (Neilson and Terry, Amer. Journ. of Physiol., 1908, **22**, 43).

Cole came to the conclusion that anions facilitate and cations weaken the action of amylase, the effects increasing with the electro-affinity (this magnitude is evidently meant by Cole, who uses the somewhat indefinite expression " actinising power ") of the ions.

The amylolytic action of pancreatin is also accelerated by a number of salts (Preti, Biochem. Z., 1907, **4**, 1) if these are added in dilute solution.

Inhibiting effects on amylase are, however, produced by calcium and barium chlorides and by larger quantities of the sulphates, phosphates and alums of the alkali metals.

According to F. Krüger, NaCl, KCl, NH_4Cl, $CaCl_2$ and $MgCl_2$, in equivalent proportions exert equal retarding effects, so that it must be concluded that the inhibiting action of the anion predominates. This recalls the concordant results of J. Schütz (Hofm. Beitr., 1904, **5**, 406) and Levites (H., 1906, **48**, 187) which indicate that peptic digestion is retarded principally by the anions.

Kudo found that the digestive action of pancreatin is, in general, weakened by alkali salts, sodium chloride producing a rather greater effect than the nitrate or nitrite; the influence of potassium salts is less than that of sodium salts.

Sodium and potassium sulphates retard the rennetic action of animal chymosin in proportion to their quantity (if the clotting effect is determined with fresh milk), whilst the coagulating action of vegetable rennet is increased by small doses, and diminished by larger ones, of these salts.

This different behaviour of vegetable and animal rennet towards neutral sulphates and towards Na_2HPO_4 and K_2HPO_4, Gerber explains as due to the precipitation of lime by these salts, lime being less necessary with the vegetable than with the animal enzymes. By small quantities of acid sulphates, such as $KHSO_4$, both animal and vegetable rennets are accelerated.

Concerning the action of activators in general, it may be said that:

As far as the "kinase" of tryptase is concerned, this can be regarded, on the one hand, as a catalyst of the reaction trypsinogen→trypsin and, on the other, as functioning like the activating acids.

It is highly desirable that a more extended series of experiments should be made to decide if the conversion of pro-enzyme into enzyme is a reversible process.

There still remains the possibility of a chemical reaction occurring between zymogen and kinase and in order to obtain information on this question, the manner in which the activation varies with the time would have to be studied more closely. That the process is a relatively rapid one has been shown by Pawlow and his collaborators.

In one way or another, the zymogen is often activated "spontaneously." In most cases the substrate or some foreign substance succeeding it yields the activator as the result of a slow reaction of some kind. In an interesting investigation Hoyer (loc. cit.) observed the appearance of lactic acid as such an activating substance. If the activator is a normal product of the substrate under the given conditions, the well-known case of auto-catalysis presents itself. A typical example of such a reaction is the spontaneous decomposition of ethyl acetate in aqueous solution; in this instance the liberated acetic acid is the catalyst which accelerates the subsequent hydrolysis in proportion to the concentration of its hydrogen ions.

Meanwhile there are no grounds for making an essential distinction between the activation of the zymogen with initiation of the reaction and the action of acids, alkalis and many salts in accelerating the reaction.

The action of the latter substances—often termed co-enzymes—rests undoubtedly on the reversible formation of compounds of these activators, partly with the substrate and partly with the

enzyme. In most cases it is a salt-formation which takes place (Euler, Hofm. Beitr., 1905, **7**, 1); this occurs instantaneously, so that the "incubation periods" of the accelerating alkalis, e.g., with catalase, or those of the inhibiting acids are without influence—of course, only so long as disturbing secondary reactions, decomposing chemically the enzyme or substrate, are avoided.

In many cases the minimal concentration determining the optimum of acid- or alkali-action must correspond exactly with the quantity of acid or alkali necessary for the neutralisation of the solution (cf. Cole, Journ. of Physiol., 1903, **30**, 202).

The sensitiveness of the enzymes towards acids and alkalis is, indeed, very great but is quite conceivable if the very small concentrations in which the enzymes themselves are present in solution are considered. More or less complete analogies are found with many well-known catalytic processes. Thus, hydroquinone in a solution containing 0·001 normal-manganese sulphate undergoes oxidation very slowly; but if sufficient alkali is added to combine with the majority of the sulphuric acid and thus to liberate manganese hydroxide, the oxidation is enormously accelerated. The manganese sulphate corresponds with the enzyme in its inactive state, the alkali with the activator. In hydrolytic changes, e.g., the enzymic inversion of cane-sugar, the chemical reaction presumably consists in the activating acid liberating the enzyme—which in neutral solution is present as a salt—and so bringing it into the active condition (cf. Euler and B. af Ugglas, H., 1910, **65**, 124).

As well as to salt-formation, an important part in the activation of enzymes must be attributed to the formation of complex compounds. It is in this way that the specific properties of phosphoric acid and of calcium and manganese are exerted, as described above. With manganese the capacity to form complex compounds with hydroxylic bodies has been long known, and with calcium, physico-chemical investigations have rendered necessary the assumption that it also yields such complexes. The reactivity of phosphoric acid with polyhydric alcohols has often been studied qualitatively, and quantitative experiments would be of biological interest.

In the description of pepsin action on p. 95, it was mentioned that hydrochloric acid not only acts on the enzyme but also accelerates the digestion by forming salts with the proteins.

Similarly, it is doubtless the alkali salts of peptones and peptides which constitute the active molecules of tryptic digestion. This complete alteration of the reactivity of a substance by salt-formation has many chemical analogies: nitrous acid diazotises, whilst nitrites do not ; in alkaline solution polyphenols are oxidised by the oxygen of the air with great readiness, but in acid solution only with difficulty. The velocity of decomposition of hydrogen peroxide is influenced by the acids or alkalis present in a similar manner, no matter whether the reaction is brought about by " catalases " or by inorganic oxides (colloidal platinum, ferric hydroxide).

A similar argument can be applied also in other cases, e.g., that of invertase-action, where it may be assumed that the compound (cane sugar-mineral acid), which represents the active molecule in the process of inversion, is resolved catalytically by the enzyme into glucose, fructose and free acid.

Being amphoteric electrolytes and hence capable of forming salts with either acids or alkalis, the proteins, peptones and amino-acids often exert indirectly an accelerating, though seldom a powerful, action; as the author has already emphasised (Ergeb. der Physiol., 1907, **6**), they regulate the concentration of the free acid or base.[1]

Finally, those cases must be considered where the activator represents the common solvent, that is, the connecting link between enzyme and substrate, where the two alone cannot form a homogeneous system. The activation of the lipases by the bile acids is possibly to be explained in this way. The purely chemical (not enzymic) splitting of fats by naphthalene-stearosulphonic acid has thus been interpreted by Twitchel.

The causes underlying the actions of neutral salts are also very varied.

These actions are partly due to simple chemical transpositions between the electrolytes present and the consequent alteration of the acidity or alkalinity. So that sodium sulphate, salicylate and phosphate hinder peptic digestion (Pawlow, Danilewski), the strong hydrochloric acid being replaced

[1] In many instances the addition of proteins to an enzyme solution mitigates the harmful action of tryptic ferments on the enzyme. Beneficial effects of another type are also observed, e.g., with diastase, ptyalin, etc.

by the weaker, less active acid of the salt. With higher concentrations of the salts, the influence of dissociation comes into play, sodium chloride diminishing the electrolytic dissociation of the protein hydrochloride. Further those influences come into action which produce the "neutral salt action" in non-enzymic hydrolyses and which vary widely in different reactions.

The actions of small quantities of neutral salts, such as NaCl, KCl, etc., would indeed seldom be observable if the salt were added to enzyme solutions previously free from electrolytes. Many investigations indicate that the small quantities of salts occurring in the organs with the enzymes are essential for the activity of the latter; if these small amounts of salts are removed by dialysis, the enzyme action, for example, of amylase, ceases.

Very large quantities of salt have a coagulating action and precipitate enzymes more or less directly from enzyme solutions, which usually contain colloidal substances. Yet even quite considerable concentrations of salts are often without harmful effect; thus the activity of ptyalin solutions is not weakened by large proportions of magnesium and ammonium sulphates[1] (Patten and Stiles, Amer. Journ. of Physiol., 1906, **17**, 26).

In addition to the reversible influence on the time-course of enzyme reactions, acids, alkalis and salts exert also an influence on the irreversible changes of the enzyme-substance. These two actions are essentially different. The latter of the two has been studied in the case of invertin by Fernbach (Recherches sur la sucrase, Thesis, Paris, 1890). This investigator found that dissolved invertin, exposed to the oxygen of the air, gradually undergoes oxidation and becomes permanently inactive; this oxidation occurs far more rapidly in alkaline than in acid solution. (Cf. also Hudson and Paine, Journ. Amer. Chem. Soc., 1910, **32**, 774).

[1] It is regarded as unnecessary to mention the many cases in which smaller proportions of salts have no marked effect.

PROTECTIVE AGENTS

Lastly, the influence of neutral salts on enzyme reactions makes itself felt in an indirect way, a beneficial effect being produced by the destruction, alteration or removal of substances which may be classed together as inhibiting agents.

These causes play a part in the interesting biological phenomena accompanying fertilisation described some years ago by Jacques Loeb.

The favourable influence of certain other substances on enzyme solutions is also often due to the counteraction of the inhibiting action of the paralysors. In this way we may regard the acid salts—often termed "buffers" —and the proteins too as protective agents. The latter may, for instance, unite with part of the alkali in the case of tryptic digestion and thus protect the enzyme, especially at relatively high temperatures, from destruction (Vernon, Journ. of Physiol., 1904, **31**, 346). Also soda, calcium carbonate, etc., which are able to protect yeast from the poisonous effect of various substances, do so in virtue of their neutralising action (Henneberg, Centralbl. f. Bakt., 1908, II, **20**, 225).

INHIBITING AGENTS (PARALYSORS)

The term "poison," which has recently been largely used for all substances which delay or prevent catalytic reactions, is not justifiable in the light of recent knowledge. The expression poisoning must therefore be reserved for the disturbance of the life functions by paralysors.

Concerning the chemistry of the action of paralysors we are almost completely in the dark, but these bodies are of considerable interest in enzymology since they are indispensable as sterilising agents in all protracted experiments. The influence which paralysors, such as chloroform etc., may at times exert on enzymic decompositions is shown, for example, by E. Fischer's observations on the hydrolysis of glucosides by yeast-enzymes (Chem. Ber., 1895, **28**, 1436).

Just as with the action of activators, that of poisons and other inhibiting substances is dependent on the concentration of the enzyme and the purity of the solution. It is found that the

injurious action of poisons on enzyme solutions increases as the concentration of the enzymes diminishes; this behaviour seems to indicate that an addition of the poison to the enzyme takes place.

Inorganic Salts

NaF. According to Arthus and Huber (C. R., 1892, **115**, 839) this salt is without influence on the soluble enzymes, but kills bacteria. Its effect on lipase is greater than that of any other antiseptic (Loevenhart, Journ. of Biol. Chem., 1907, **2**, 391; Kastle and Loevenhart, Amer. Chem. Journ., 1900, **24**, 491). Chymosin is injured by it, but not stronger solutions of trypsin (Kaufmann, H., 1903, **39**, 434). The action of erepsin on dipeptides is partly retarded, partly accelerated by sodium fluoride (Abderhalden, Caemmerer and Pinkussohn, H., 1909, **59**, 293). According to Vandevelde it has no effect on pepsin and trypsin. Buchner states that ammonium fluoride annuls the action of zymase. The influence of fluorides on thrombin has been thoroughly investigated by Bordet and Gengou (Ann. Inst. Pasteur, 1904, **18**, 98) and that on diastases by Effront.

$HgCl_2$: even in 0·00005N-solution has a harmful effect on catalase. In 0·001% solution, it is poisonous to amylase and still more so to urease. It injures ptyalin or trypsin in 0·005% solution, but its action on erepsin is much less marked (Euler, Arkiv för Kemi, 1907, **2**, No. 39). Invertin is also weakened by it, but to a relatively small extent (Duclaux).

$Hg(CN)_2$: exerts a decided inhibiting action, although less than that of $HgCl_2$, on catalase (Faitelowitz).

$B(OH)_3$. Duclaux found that this retards the action of chymosin, but the more recent results of Agulhon (C. R., 1909, **148**, 1340) indicate an accelerating effect. According to the latter author, the enzymes which hydrolyse carbohydrates, glucosides and proteins act, without alteration, in cold saturated boric acid solution; catalase is somewhat retarded.

As_2O_3. As Buchner has shown by an extended series of experiments, arsenic is injurious to cell-free fermentation, but proteins and sugar act as protecting agents against this

poison. Amylase (Kjeldahl) and pepsin (Asher) are harmfully affected by arsenic salts.

H_2S: has an injurious influence on catalase but is without action on pepsin, trypsin, diastase and emulsin (Fermi and Pernossi, Zeitschr. f. Hygiene, 1894, **18**, 83).

O_3 (ozone). Whilst hydrogen peroxide accelerates many enzyme actions and, so far as is known, has a slow destructive action only on catalase, ozone has a harmful influence, as has been shown by Sigmund for most of the more important enzymes, by Kastle especially for lipase (Journ. Chem. Soc., Abs., 1906, i, 615) and by Buchner and Hofmann for zymase (Biochem. Z., 1907, **4**, 215).

H_2O_2: according to Vandevelde (Hofm. Beitr., 1904, **5**, 558), most enzymes are beneficially affected, only catalase being retarded.

Of salts which have a more specific injurious action, mention may be made of the following:

Alkali sulphates retard peptic digestion, as was found by Grützner (Pfleiderer, Pflüg. Arch., 1897, **66**, 605) (with trypsin there is either slight retardation or no effect at all).

$CaCl_2$ has an especially marked weakening action on invertin (Duclaux).

Iron salts are injurious to pepsin (Asher).

Potassium permanganate strongly inhibits lipase (Kastle and Loevenhart).

Nitrates and chlorates are stated to be intense catalase-poisons.

For certain other substances which hinder the action of blood-catalase, Senter (Zeitschr. f. physikal. Chem., 1905, **51**, 673) gives the following concentrations as necessary to diminish the velocity of reaction to one-half its original value:

Paralysor	Grm.-mol. per litre
I_2 in KI	1/50,000
Hydroxylamine hydrochloride	1/80,000
KNO_3	1/40,000
$KClO_3$	1/40,000

For the catalase from frog's muscle, C. G. Santesson (Skand. Arch. f. Physiol., 1909, **23**, 99) has recently obtained similar results.

Inorganic colloids (gold, platinum, silver, arsenic, copper, mercury, bismuth) do not accelerate pepsin, but in high concentrations rather inhibit it (Pinkussohn, Biochem. Z., 1908, **8**, 387).

Organic Poisons and Inhibiting Agents

Chloroform. The original statement made by Müntz, that chloroform injures only the micro-organisms but not the enzymes, has had to be considerably modified in recent times.

Chloroform injures maltase (according to Lintner and Kröber), amylase, ptyalin, yeast-glucase, pepsin, rennin and urease, but has no, or but slight action on trypsin, erepsin, invertin (Fischer), Aspergillus-maltase (Hérissey) or zymase. For the literature on this subject see Kaufmann (H., 1903, **39**, 434).

Chloral completely destroys oxydase (from Lepiota americana) (Kastle and Loevenhart, Chem. Zentralbl., 1906, **77**, i, 1554) but injures myrosin only slightly.

Formaldehyde. In 40% concentration, this does not destroy Lepiota-oxydase (Kastle and Loevenhart, loc. cit.) but it has an injurious effect on chymosin and amylase. In 1% solution, it is without action on erepsin (Euler, loc. cit.). Zymase is harmfully influenced by formaldehyde but pepsin only by concentrated solutions.

Glycerol has an appreciable inhibiting influence on rennet-action (Reichel and Spiro, Hofm. Beitr., 1905, **7**, 485).

Toluene. This hydrocarbon, which E. Fischer introduced for the sterilisation of enzyme solutions, is harmless in the great majority of cases, but urease is weakened by it.

Phenol: exerts a deleterious action on pepsin, amylase and catalase.

Cresols: harmless for liver-butyrase (Kastle, Chem. Zentralbl., 1906, **77**, i, 1555).

Thymol: injurious to oxydases (Kastle and Loevenhart) and saliva-diastase (Schlesinger, Pugliese) and markedly so to zymase (Buchner) and chymosin (Freudenreich). It does not weaken the action of the more

concentrated trypsin solutions (Kaufmann, H., 1903, **39**, 434) or that of yeast-maltase (E. Fischer).

Maltose retards peptic digestion (Sailer and Farr).

Salicylic acid: stated to have a weakening effect on pepsin and trypsin and also on lipase. Autolytic action and that of xanthine-oxydase (Burian, H., 1905, **43**, 494) are, however, accelerated.

Hydrocyanic acid. Whilst this acid has proved itself an extremely powerful poison towards catalase, its deleterious action on other enzymes is considerably weaker and sometimes very faint. Zymase is, indeed, completely inactivated, but this action is reversible (Buchner). Also, according to Fuld and Spiro, chymosin is not injured and the same is the case with pepsin. The decomposition of polypeptides by erepsin is accelerated by small, but inhibited by larger, quantities of potassium cyanide (Abderhalden, Caemmerer and Pinkussohn, H., 1909, **59**, 293). In 1% solution, hydrocyanic acid weakens but does not destroy the proteolytic enzyme of yeast-juice (Geret and Hahn, Chem. Ber., 1898, **31**, 202).

The sensitiveness of catalase towards hydrocyanic acid is shown by Senter's results which, with those obtained with certain other organic poisons are given below.[1] The concentrations required to diminish the velocity of reaction to one-half are:

Paralysor.	Grm.-mol. per litre.
HCN	1/1000000
Phenylhydrazine	1/20000
Aniline	1/400

Alkaloids. The older literature on this subject is given by Nasse (Pflüg. Arch., 1875, **11**, 159) and in the work of

[1] Faitelowitz has arranged various paralysors, according to the concentrations in which they affect milk-catalase, in the following order:

I	II
HCN	$H_2C_2O_4$
KCN	HNO_3
KCNS	$Ba(NO_3)_2$
$HgCl_2$	HCl
H_2S	CH_3COOH
$Hg(CN)_2$	

Chittenden, to whom a very thorough investigation is due. In general, alkaloids retard enzyme action, but not very strongly (Chittenden; Gockel); cf. also Laqueur (Arch. f. exp. Path., 1906, **55**, 240) who studies especially the effect of quinine on enzymes. According to the latter author, the autolytic enzyme and blood-oxydase are the most strongly inhibited by quinine. Asher observed a deleterious action of quinine preparations on peptic digestion. Oxydases appear to be especially sensitive to alkaloids (Rosenfeld). Hordenine sulphate retards peptic and tryptic digestion (Camus), but not the action of maltase, invertase or lipase.

Of practical importance for the technique of enzymology are the investigations dealing with the action of alcohol on enzymes:

Small quantities of alcohol have an accelerating influence on lipase (Gizelt, Zentralbl. f. Physiol., 1905, **19**, 769, 851) but in other cases a more or less complete inhibiting action, e.g., on trypsin (Gizelt, loc. cit.), rennet (Reichel and Spiro, Hofm. Beitr., 1905, **7**, 485) and diastase. Only tyrosinase is able to act in 50% methyl-ethyl alcohol. Inhibition by alcohol is almost always reversible.

Even very large quantities of alcohol are withstood for a time by all enzymes, as the ordinary precipitation methods show. If the alcohol is removed after precipitation of the enzyme, the latter resumes its activity (Schöndorff and Victorow, Pflüg. Arch., 1907, **116**, 495).

Like the activators, inhibiting agents exert their action by combining partly with the substrate, partly with the enzyme and partly with the activator. Especially often where the paralysors are acids or bases must these actions play a part. Also, salts of the heavy metals alter the state of solution of colloidal substrates such as proteins, starch, etc.

The behaviour exhibited by antiseptic and narcotic media, such as toluene, thymol and chloroform, towards living cells is determined principally by the behaviour and the alterations of the lipoidal plasma-skin. The plasma undergoes change only after the penetration of the plasma-skin by the narcotic.

The enzymic actions of living cells are influenced in various ways. By toluene or chloroform, fermentation, for example, is interrupted, although zymase itself—as is shown by experi-

ments with press yeast-juice—is not injured by chloroform. On the other hand, yeast-cells invert cane-sugar equally quickly in absence or presence of chloroform or toluene.

H. Euler and Beth af Ugglas (H., 1911, **70**, 279) have arrived at the result, that the activity of those enzymes which are combined with the plasma in the living cell, e.g., zymase, is annulled by narcotics, whilst the enzymes occurring free in the cells, as, for example, invertase, are not influenced by these substances.

Euler and Kullberg have investigated the connection between the sensitiveness to poisons, extractability, and relative quantities of the enzymes of a beer-yeast called "H" from a Stockholm brewery (H., 1911, **73**, 85), the results being given in the following table:

		Zymase.	Monilia-invertase.	Maltase.	Beer-yeast invertase.
Living yeast	Relative velocity of reaction in 8% sugar solution	1	1 (to 2)	1 (to 2)	170
	Extractability...	0	0	0	slight
	Action of poisons:				
	Chloroform.....	inhibited completely	inhibited completely	inhibited completely	scarcely any weakening
	Thymol.....	ditto	ditto	considerable weakening	no weakening
	Toluene.....	ditto	ditto	considerable weakening	ditto
	Weakening on drying.....	20 : 1	25 : 1	—	2 : 1
Dried yeast	Extractability...	very slight	very slight	very incomplete	about 20%
	Action of poisons:				
	Chloroform...	weakened	—	—	not weakened
	Toluene.....	weakened	weakened	weakened	not weakened

Concerning the chemical compounds presumably formed in the poisoning of the free enzymes we have no data, since we do not even know the nature of the reacting enzymic substance. These chemical combinations often appear to be only loose ones; the ability of the enzyme to regain wholly or partially its original activity after removal of the paralysor, is especially marked with "poisoning" by hydrocyanic acid.

If the paralysor is present in only very small quantities, it may be destroyed by any oxidising agent present or by other enzymes without external action. To this must be attributed the spontaneous re-activation which is known as "recovery."

Analogies to known chemical processes are here also not lacking. A comparison has often been drawn between the actions exerted by paralysors on catalase and on Bredig's so-called "colloidal metals"—which are, indeed oxides—and other oxidising catalysts. But even Senter's thorough experiments, referred to above, are not able to explain to what chemical changes the active oxygen, the action of which is here in question, is subjected by the paralysors. Besides, the "poisonings" with catalase and colloidal platinum follow by no means parallel courses. The principal result of the interesting contributions of Höber (Pflüg. Arch., 1900, **82**, 631) and of Loevenhart and Kastle (Amer. Chem. Journ., 1903, **29**, 397) to this subject may be summed up in the sentence: "That the effect of any particular substance on the catalyser can be explained, in the majority of cases at least, upon purely chemical grounds."

In many respects the anti-bodies correspond with the inhibiting substances here considered and the limits of the term anti-bodies are determined by characteristics somewhat similar to those which define the limits of the enzymes in the wider field of the catalysts. A number of substances which retard enzymic reactions in the normal organism should also be termed inhibiting substances in contradistinction to the anti-enzymes, which the organism forms as protective substances after the introduction of foreign enzymes.

A whole group of inhibition phenomena are to be attributed to the adsorption of enzymes. To such phenomena belongs especially the inhibition of trypsin, rennet and invertase by charcoal, studied by S. G. Hedin and by A. Eriksson. Hedin showed that the retardations caused by white of egg and serum-albumin are analogous to those produced by charcoal, that is, they are due to adsorption phenomena. To this group belong therefore all the non-specific inhibiting effects of serum which were formerly ascribed to anti-bodies. Hedin has collected the literature on this subject in the ninth yearly volume of the Ergebnisse der Physiologie (1910) (cf. H., 1911, **72**, 313).

The data are, however, too incomplete to permit of a critical classification of these inhibiting substances, so that a résumé of all substances referred to in the literature as anti-enzymes will be given in a later chapter.

Reference must finally be made to those inhibiting effects observed when heated enzyme solutions are added to the active enzymes.

Such retardation has been observed:

With tryptase by Pollak (Hofm. Beitr., 1904, **6**, 95) in the digestion of gelatine.

With peptase by Schwarz (Hofm. Beitr., 1905, **6**, 524), according to whom the inhibitory substance resists the action of heat and exists ready-formed in fresh solutions of the enzyme.

With peptase, rennet and taka-diastase by Cramer and Bearn (Proceedings of the Physiol. Soc., June 2, 1906, see Journ. of Physiol., 1906, **34**, xxxvi; Biochem. J., 1907, **2**, 174), who heated the enzyme solutions to 50–60°; at 100° the inhibiting substance is destroyed.

With invertase by A. Eriksson (H., 1911, **72**, 330), who assumes the existence of an inhibiting agent, resistant to heat, in invertase solutions.

Here belong also the results obtained by Porter (Biochem. Z., 1910, **25**, 301) with peptase, tryptase, rennet, lipase, saliva-amylase, amygdalase and taka-diastase. In contact with collodion membranes these enzymes, with the exception of taka-diastase, lose their activity, all of them except saliva-amylase then exhibiting a retarding action on the corresponding enzymes.

CHAPTER IV

CHEMICAL DYNAMICS OF ENZYME REACTIONS

The relations required by chemical dynamics for the simplest cases of catalytic reactions are found to be fulfilled by enzymic processes to very varying extents. In some cases the time-law for unimolecular reactions and the proportionality between velocity of reaction and amount of catalyst are closely followed. But in the majority of instances the experimental data are in agreement with the doctrine of reaction only within a limited region of concentration, and not a few reactions will be described for which no simple theoretical representation has yet been found possible.

The arrangement of the various enzymic processes according to the mathematical expressions which they follow is apparently the simplest method to adopt. But this can only be carried out at the expense of brevity and clearness since, as already mentioned, the kinetics of the action of one and the same enzyme may be altered completely by change of the external conditions. Separate treatment will therefore be given for each of the more important enzymes, beginning with the hydrolysing enzymes and passing on to the fermentation enzymes, oxydases and catalases.

The opportunity must not be neglected of emphasising the necessity of a critical examination of the numerical data of the quantitative results given below. Even in the most favourable cases, where a chemically individual substrate has been employed, the solutions dealt with not only contained a chemically unknown catalyst in unknown concentration, but were also contaminated with the foreign constituents of the enzyme preparation and, indeed, with substances which, sometimes even in minimal amounts, might exert a deciding influence on the course of the reaction to be observed. In short, one is in the doubtful position of making

quantitative observations on a system insufficiently investigated qualitatively.

It might then be asked: Are quantitative results obtained with enzyme solutions of any value at all and is any detailed treatment of them advisable? If the observations made on a preparation are not very comprehensive and if the experimental conditions are varied only inconsiderably, the value of a physicochemical investigation of an enzyme is, indeed, small. But our knowledge of enzymes has been widely extended by a number of studies of the kinetics of reaction, which, especially when considered as a whole, have elucidated the general relations of enzyme action and have furnished valuable aid in the elaboration of enzymological methods.

Before the experimental results are examined in detail, it will be well to consider the theoretical principles to be applied in judging these results.

THEORETICAL PRINCIPLES OF ENZYMIC DYNAMICS

A knowledge of the law of mass action may be assumed. This states that the active mass of a substance is proportional to its osmotic pressure and hence (within rather narrower limits) to its concentration. If a single type of molecule A with a concentration C_A is changed by a chemical reaction into new substances, without the concentration of any other kind of molecule present being appreciably altered, then—for given external conditions of temperature, pressure and medium—the quantity of substance dC_A changed in every interval of time dt per unit of volume will be given by the equation:

$$v=\frac{dC_A}{dt}=k'C_A, \quad \ldots\ldots \quad (1a)$$

where k' is a constant, termed the velocity constant (also reaction constant) of the process.

This constant k' retains—and on this emphasis must be laid—its value undiminished, no matter how far the reaction has proceeded or what initial concentrations may be chosen.

On the other hand, the velocity v, that is, the amount of substance changed per unit of time and in unit volume, is

dependent on (proportional to) the concentration C_A. It assumes the same numerical value as k', if $C_A = 1$, i.e., if the substance transformed amounts to one grm.-mol. per litre and is in some way maintained at this concentration.

This simplest equation allows, therefore, of the representation of a chemical process which proceeds (practically) completely in one direction.

Integration of (1*a*) gives the constant for so-called unimolecular reactions:

$$k' = \frac{1}{t} \cdot ln \frac{a}{a-x} \quad \text{or} \quad k = \frac{1}{t} \cdot \log \frac{a}{a-x}, \quad . \quad . \quad . \quad (1b)$$

if we indicate decimal logarithms by log and hence make $k = 0 \cdot 4343\ k'$.[1]

An example of an enzyme reaction in which molecules of only one kind undergo change, is the decomposition of hydrogen

[1] If a reaction proceeds so that equimolecular quantities of two substances combine to form the product of the reaction, i.e., according to the scheme,

$$A + B \rightarrow C,$$

then, if the initial concentrations are a and b, and x denotes the amounts of these two substances (and hence the concentration of C) changed after time t, the velocity at any moment is proportional to the concentrations of the two reacting substances; thus,

$$\frac{dx}{dt} = k(a-x)(b-x),$$

or, if the two substances have originally the same comcentration, a,

$$\frac{dx}{dt} = k(a-x)^2.$$

This, the simplest case of a so-called bimolecular reaction, corresponds with the integral

$$k = \frac{1}{at} \cdot \frac{x}{a-x}.$$

Enzymic processes proceeding according to the equation for bimolecular reactions, are as yet unknown.

peroxide by catalase; the tables given on p. 216 show that the values of k are constant within the limits of experimental error.

The above equation is also confirmed in numerous cases where, in addition to a dissolved substrate, water takes part in the reaction. Since the water is usually present in large excess compared with the dissolved substance, its concentration may be regarded as constant; so that here also only molecules of a single kind undergo change. Indeed the first example of the validity of the unimolecular reaction law was that of the inversion of cane-sugar by acids (Wilhelmy, 1850). Hudson's recent measurements, the results of which are given on p. 160, show that the same law holds for the hydrolysis of this sugar by invertase.

It may here be pointed out that the law of mass action, on which the whole of chemical kinetics depends, may be derived from the two fundamental laws of thermodynamics, and is hence independent of our present molecular kinetic conceptions. That the law of mass action underlies all chemical processes, cannot therefore be doubted; the only question is as to when and how far the assumptions, according to which it can be expressed in the above simple form, are valid. If we find deviations from the simple formulæ to which the law of mass action leads, we have to enquire which of the assumptions are not fulfilled under the experimental conditions chosen.

CATALYSIS

The hydrolysis of cane-sugar, as is well known, proceeds with extreme slowness in pure water; the velocity is considerably increased only when the solution contains an acid (or an enzyme, invertase), in addition to the sugar. So far as we can determine by titration, the concentration of the added acid does not change during the hydrolysis. Also the law for unimolecular reactions holds equally well for a solution containing either 0·001 or 0·1 grm.-mol. of acid per litre, the sole change (so long as we remain within the region of dilute aqueous solutions) being in the numerical value of the reaction constant k. A catalyst is therefore, as mentioned at the outset, to be defined as a substance which, without being consumed in the reaction, alters the velocity with which a

reaction attains its position of equilibrium.[1]

In 1884 Arrhenius made the important discovery, that the catalytic action of different acids runs parallel with their conductivity or, more accurately, with the amount dissociated.

This law, which has been often confirmed and is of the widest significance, can be expressed by saying that different acids catalyse hydrolytic reactions in proportion to the concentration of the hydrogen-ions of their solutions. This fact is often stated in the literature in such a way as to imply that the hydrogen-ions alone are the catalysing agent and that they function as a kind of contact-substance. But such a representation by no means corresponds with the chemical facts. Rather must it be supposed that, by the catalysing acid the concentration of the ions effecting the reaction is increased (Euler, Zeitschr. f. physikal. Chem., 1901, **36**, 681)

The supposition that combination of the catalyst with the substrate yields the molecules which carry on the reaction, has already received general acceptance in enzymology. Of the authors who, on the basis of their own investigations, have expressed themselves in this sense, mention need only be made of: Kastle and Loevenhart, Bach, Hanriot, A. Brown, H. Brown and Glendinning, Bodenstein, Henri, Medwedew, Hedin, Armstrong and Bayliss.

In many enzymic reactions, compounds between enzyme and substrate seem to occur to a far greater extent than is the case in catalytic hydrolyses by acids; yet in no instance has it been determined what proportions of the total quantities of enzyme and substrate present combine during the reaction.

It would lead too far to indicate the reasons which have caused the various investigators to assume a combination of enzyme with substrate. We shall only indicate briefly the mathematical formulation of the hypothesis in question.

In his researches on the inversion of cane-sugar, Henri found that the reaction constants of the first order increased considerably

[1] This holds for all those cases where a substance does not catalyse its own transformation, as is, for example, the case with the formation of lactones from γ- and δ-hydroxy-acids, the hydroxy-acid here accelerating the lactone-formation proportionately with the amount of its dissociated portion (auto-catalysis).

(cf. p. 158), and, in order to obtain a mathematical expression of his results, he employed (Zeitschr. f. physikal. Chem., 1902, **39**, 194) a method given by Ostwald (Lehrbuch der allgem. Chemie, II, **2**, 265).

If a reaction is accelerated by a substance having the concentration p, the expression $k(a-x)$ is to be multiplied by $(1+\varepsilon p)$, ε being a constant.

For the case in which the product of the reaction is the accelerating agent, the velocity will hence increase in the ratio $1:\left(1+\varepsilon\frac{x}{a}\right)$, i.e., in proportion to the transformed part $\frac{x}{a}$ of the substrate. The equation therefore becomes:

$$\frac{dx}{dt}=k_H\left(1+\varepsilon\frac{x}{a}\right)(a-x). \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (1c)$$

On integration, this gives:

$$k_H(1+\varepsilon)=\frac{1}{t}\left[\log\frac{a}{a-x}+\log\left(1+\varepsilon\frac{x}{a}\right)\right], \quad . \quad . \quad . \quad . \quad (1d)$$

or

$$k_H(1+\varepsilon)=\frac{1}{t}\log\frac{a+\varepsilon x}{a-x}. \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (1e)$$

Calculation of several series of experiments on the action of invertase gave, for the new constant ε, values approaching 1. Thus, if $\varepsilon=1$, the equation becomes

$$2k_H=\frac{1}{t}\left[\log\frac{a}{a-x}+\log\left(1+\frac{x}{a}\right)\right],$$

or

$$2k_H=\frac{1}{t}\log\frac{a+x}{a-x}. \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (2)$$

Shortly afterwards Bodenstein subjected Henri's numbers to a re-examination, making the assumption that both the cane-sugar a and its hydrolytic products weaken the invertase, the former more strongly than the invert-sugar. From this he derived the formula:

$$k_BE=\frac{a+i}{t}\left[(m-n)\frac{x}{a+i}+n\log\frac{a}{a-x}\right]. \quad . \quad . \quad . \quad (3)$$

(Cf. Henri, Lois générales de l'action des diastases, Paris, 1903, p. 77 et seq.)

Here i indicates the quantity of invert-sugar previously added, E denotes the quantity of enzyme and m and n are constants expressing the specific weakening of the invertase by cane-sugar and invert-sugar respectively. Bodenstein chose for these constants the values

$m=2$ and $n=1$, thus indicating the stronger action of the cane-sugar. If the solution originally contains no reaction-product ($i=0$), his formula simplifies to the following:

$$k_B E = \frac{a}{t}\left[\frac{x}{a} + \log\frac{a}{a-x}\right]. \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (4)$$

While this equation corresponds satisfactorily with observations on moderately dilute solutions, the numbers obtained by Henri with dilute solutions are not in agreement with it.

Henri has therefore deduced another expression on the assumption that both the cane-sugar and the invert-sugar (especially the fructose) combine with the enzyme (Lois générales, p. 85 et seq., and C. R., 1902, **135**, 916).

Of the original quantity of substance a let x molecules be hydrolysed, so that $a-x$ molecules remain. Further, let the quantity of enzyme be E and X the quantity of it which is free and Z and Y the amounts which, at the time t, are combined with the cane-sugar and invert-sugar respectively.

Between enzyme and substrate on the one hand, and enzyme and products of reaction on the other, equilibria must set in in accordance with the law of mass action. Hence for these two equilibria the following relations hold (in the case where 1 mol. of enzyme unites with 1 mol. of substrate):

$$X(a-x) = \frac{1}{m}\cdot Z, \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (5)$$

and

$$Xx = \frac{1}{n}\cdot Y, \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (6)$$

where m and n are equilibrium constants.

Further, for the total quantity of enzyme

$$E = X + Y + Z. \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (7)$$

From these Henri calculated the quantity of free enzyme X and that of the compound sugar-enzyme Z:

$$X = \frac{E}{1+m(a-x)+nx}, \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (8)$$

and

$$Z = \frac{mE(a-x)}{1+m(a-x)+nx}. \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (9)$$

Only two assumptions can now be made:

1. The free portion of the enzyme acts on the sugar; in this case the velocity is proportional to the quantity of free enzyme and to the quantity of cane-sugar, i.e., to X and to $a-x$. Hence

$$\frac{dx}{dt}=\text{const. } X(a-x). \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (10)$$

If X is substituted in accordance with Eq. (8), this gives

$$\frac{dx}{dt}=\frac{\text{const. } E(a-x)}{1+m(a-x)+nx}. \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (11)$$

2. If, on the other hand, the reaction is effected by the complex, enzyme-sugar, the velocity will be proportional to the concentration of these molecules, i.e., to Z. From

$$\frac{dx}{dt}=\text{const. } Z,$$

is obtained, from Eq. (9),

$$\frac{dx}{dt}=\frac{\text{const. } m.E(a-x)}{1+m(a-x)+nx}. \quad . \quad . \quad . \quad . \quad . \quad . \quad (12)$$

So that both assumptions lead to one and the same expression for the velocity of reaction.

On the supposition that complexes are formed consisting of 1 mol. enzyme, p mols. of substrate and q mols. of the various products of the reaction, Arrhenius (Immunochemistry, p. 60) gives a still more general form for the above expression.

For every single such molecule a formula is obtained of the form:

$$Z=\kappa' X(a-x-\Sigma pZ)^p(x-\Sigma qZ)^q, \quad . \quad . \quad . \quad . \quad . \quad (13)$$

where

$$E=X+\Sigma Z.$$

If we assume that ΣZ is small compared with a and x, we obtain

$$Z=\kappa' X(a-x)^p x^q,$$

so that

$$\frac{dx}{dt}=\kappa' X(a-x-\Sigma pZ)^a=\frac{\kappa' E(a-x)^a}{1+\Sigma\kappa'(a-x)^p x^q}. \quad . \quad . \quad . \quad (14)$$

If the first term of the denominator, 1, is small with respect to the terms under the summation sign, Bodenstein's formula is obtained if we make $a=1$ and assume that there are two terms under the Σ, one in which $p=1$ and $q=0$ and a second in which $p=0$ and $q=1$.

In a preliminary communication published two years later, Henri (Zeitschr. f. physikal. Chem., 1905, **51**, 19) outlines a new theory of enzyme-action which takes account of the colloidal condition of enzymes. According to the law of distribution, the dissolved body, sugar for example, must be distributed between the aqueous solution and the colloid, the velocity of reaction being determined by the concentration of the sugar in the colloid. In this way Henri explains the similarity between the ordinary adsorption curves and those representing the influence of the concentration of the cane-sugar on the velocity of inversion by invertase.

None of these formulæ and theories have been confirmed in a manner free from objection.

As has been mentioned previously, the hydrolysis of cane-sugar, esters and other similar substances is accelerated by acids, the acceleration being proportional to the concentration of the hydrogen-ions in the solution. If, as is usually done, strong acids are used in very low concentrations, there is approximate proportionality between the concentration of the catalyst and the velocity of reaction, since the electrolytic dissociation of the acid is virtually complete.

Such proportionality between concentration of the catalyst and the velocity of reaction is found to hold in numerous enzyme reactions within quite wide limits of concentration. This is the case, for example, with the actions of the lipases (p. 146 et seq.), catalases (p. 216), invertase (p. 160) and erepsin (p. 189).

An exception to this very simple relation has, however, been known for a long time. The amounts of protein digested by pepsin in a definite time are proportional, not directly to the quantities of pepsin, but to the square roots of these. This is the law which was enunciated by Emil Schütz in 1885 (H., 1885, **9**, 577).

The validity of this rule has, indeed, often—even in recent times—been contested. But the reliability of the numerous experiments, made by different methods (cf. p. 176 et seq.) and confirming Schütz's numbers—we are referring now exclusively to pepsin action—cannot be doubted. Hence for peptic digestion, at any rate in the first third of the reaction, Schütz's rule is obeyed, and we are met with the problem of explaining this experimental relation on the basis of the doctrine of chemical dynamics.

Arrhenius (Medd. Nobel Inst., 1908, **1**, No. 9) has deduced Schütz's rule theoretically in the following way:

In order to ascertain the circumstances which condition Schütz's rule, we proceed as follows: If the amount of substance transformed is indicated by x and the time by t, the rule states that:

$$x = \kappa_1 \sqrt{t} \quad \text{or} \quad x^2 = \kappa_1^2 t. \quad . \quad . \quad . \quad . \quad . \quad (15)$$

On differentiation this gives:

$$2x dx = \kappa_1^2 dt$$

or:

$$\frac{dx}{dt} = \frac{\kappa_1^2}{2} \cdot \frac{1}{x}. \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (16)$$

So that, for Schütz's rule to hold, it is a necessary and, as may easily be seen, sufficient condition that the velocity of reaction shall be inversely proportional to the quantity of substance transformed, i.e., to x. Since the rule is only obeyed during the initial stages of the reaction, this proportionality must only be assumed for the first part of the change.

Such proportionality may be brought about as follows: The velocity of reaction—in the case when only one molecule of each of the reacting bodies goes into the product of the reaction—is proportional to the product of the concentrations of the reacting substances. At the beginning of the reaction, such small quantities of the bodies are transformed that generally their total quantities may, with sufficient accuracy, be regarded as constant. It is this that limits the validity of Schütz's rule to the beginning of the reaction. Now the active quantity (M) of one of the reacting bodies must be inversely proportional to the amount changed, i.e., to the quantity of new product (x), so that:

$$M = \frac{\text{const.}}{x} \quad \text{or} \quad Mx = \text{const.}$$

This is evidently the case if a chemical equilibrium is set up between the new product and one of the reacting bodies, on the one hand, and a compound of them in almost constant quantity, on the other. Such a case is already known in the saponifying action of ammonia on an ester, e.g., ethyl acetate. The one

reacting body here is the ion OH and the quantity of this ion (M_{OH}) is diminished by the NH_4-ions (amount, x_{NH_4}) of the ammonium acetate formed during the reaction according to the equation:

$$M_{OH} . x_{NH_4} = C_1(N_{NH_4OH} + N_{NH_3}),$$

where N_{NH_4OH} and N_{NH_3} denote the amounts of the NH_4OH- and NH_3-molecules respectively. The volume remains constant during the reaction and the last-named quantity may be regarded as constant, so long as the beginning of the reaction is alone considered.

A closer investigation of this case by Arrhenius has revealed distinctly an analogy to the course of peptic digestion.

Arrhenius considered a system in which ammonia in an initial quantity A acts on ethyl acetate, and he assumed, for the sake of simplicity, that the amount of ethyl acetate is so large that it is not changed appreciably by the reaction but may be regarded as constant (P); that is, the ethyl acetate must be present in considerable excess. If now x mols. of ammonium acetate—which with the high dilution that we assume may be considered as completely decomposed into NH_4- and CH_3CO_2-ions—are formed at the time t, $(a-x)$ mols. of ammonia will be present at the same time. Owing to the slight dissociation of ammonia, only a very small fraction of it is changed into NH_4- and OH-ions, so that it is sufficiently exact to denote the amount of non-dissociated ammonia by $(a-x)$. Even in presence of a very small quantity of ammonium salt, the NH_4-ions from the ammonia may be neglected in comparison with those formed by the salt, and the amount of NH_4-ions may be taken as x. On this assumption the first moments of the reaction must be disregarded, as then no ammonium salt or but very little is present. The concentration of the hydroxyl-ions, q, is then determined by the following equation:

$$q . x = K_2(a-x),$$

where K_2 denotes the dissociation constant of the ammonia.

The velocity of reaction on hydrolysis is now proportional to the quantity of hydroxyl-ions (q) and to the amount of ethyl acetate (P), that is:

$$\frac{dx}{dt} = \kappa_2 K_2 \frac{a-x}{x} \cdot P = \kappa P \frac{a-x}{x}, \quad . \quad . \quad . \quad . \quad (17)$$

where x_2 and x are constants and xP may be regarded as a new constant, since the value of P does not change in any single experiment

On integration, the last equation gives:

$$F(x) = a\ ln\frac{a}{a-x} - x = \kappa Pt. \quad . \ . \ . \ . \ . \quad (18)$$

The following table, taken from that given by Arrhenius, contains the result of a duplicated experiment in which a given quantity of ammonia acted on 0·66-normal ethyl acetate at 14·8°.

Mean value of $\kappa P = 21$.

t	Percentage of ammonia converted.		κP.
	Observed.[1]	Calculated.	
1	17·5	19·4	17·4
2	25·5	25·2	22·0
3	30·7	30·6	21·2
5	38·5	38·5	20·9
10	51·2	51·3	20·9
15	59·6	59·7	20·9
22	67·5	68·0	20·6
30	74·5	74·7	21·2
50	84·8	85·0	20·8
70	91·1	90·7	21·6
100	95·3	95·3	21·1

[1] Mean of two experiments.

As will be seen, formula (18) is in excellent agreement with the observed results.

The analogy between the hydrolysis of esters by ammonia and the digestion of protein by pepsin is, according to Arrhenius, as follows:

In the decomposition of protein, albumoses and peptones are formed. Most of the pepsin is fixed by the products of the reaction, so that the following equilibrium is set up:

[Free pepsin] × [products of reaction] = const. [combined pepsin].

The quantity of free pepsin is, approximately, inversely proportional to the amount of the reaction products, x. This holds as soon as so much reaction-product is formed that the

greater part of it is not combined with pepsin, whilst the greater part of the latter is combined.

Further, the amount of free pepsin is evidently proportional to the quantity of pepsin employed. Hence, if we indicate the concentration of the enzyme (pepsin) by $[E]$, the unaltered protein by $(a-x)$ and the products of digestion by x, we obtain:

$$\frac{dx}{dt}=\kappa[E]\,\frac{a-x}{x}, \qquad \ldots\ldots \quad (19)$$

and consequently, if we express the amount of hydrolytic products as fractions of 1000:

$$1000(ln1000 - ln \text{ unaltered protein})\text{—digested protein} = \kappa[E]t. \quad (20)$$

In reality, peptic digestion corresponds remarkably well with Arrhenius's formula and it is because the latter holds over wider concentration-limits of enzyme and substrate that the work of Arrhenius on Schütz's rule has been here referred to at length.

Whether, indeed, the above considerations take account of all the facts and factors essential to peptic digestion, e.g., the combination of hydrochloric acid (cf. Jastrowitz, Biochem. Z., 1907, **2**, 157), cannot at present be easily decided. In particular it is still uncertain what proportion of the pepsin combines, under definite conditions, with the substrate or reaction-products and what proportion with the hydrochloric acid. All that can be said is that the spatial configuration of the participating substances plays a very real part. This can be seen from the influence which additions of optically active bodies exert on digestive processes.

It would be of interest to ascertain the concentrations of hydrochloric acid for which pepsin-digestion is proportional to the concentration of the enzyme.

The opportunity must be taken here of pointing out that, with many enzymic reactions, the chemical change is composed of several separate processes which have not yet been studied singly; this is the case, for example, with the hydrolysis of proteins or starch by proteinases or amylases. In such complex reactions as these it cannot, of course, be expected that the laws of chemical dynamics will be apparent in their simplest form.

In addition to the above processes, the fermentation of bioses by yeasts may be mentioned; it is here assumed that the first phase consists of a hydrolysis of the bioses (cf. Euler and Lundeqvist, H., 1911, **72**, 103).

Finally, as has been done by C. Engler and R. O. Herzog (H., 1909, **59**, 327), enzymic oxidations may be represented as coupled or induction reactions. If M, N and P are three substances capable of reacting as follows:

$$M+N=(MN) \text{ react.}$$

$$P+N \text{ do not react.}$$

$$M+N+P=(MN)+(BN) \text{ react.}$$

(where the bracketed letters indicate the substances reacting with one another), the reaction is a coupled or induced one. Schilow (Zeitschr. f. physikal. Chem., 1903, **42**, 641; cf. also Luther and Schilow, Zeitschr. f. physikal. Chem., 1903, **46**, 777) terms the substance N, taking part in both reactions, the actor, M the inductor and P the acceptor. According to Engler and Herzog, the

oxydase functions as		inductor,
oxygen	"	actor,
oxidised substance	"	acceptor.

This conception opens up a number of interesting points.

REVERSIBLE REACTIONS

Influence of the Products of Reaction. Up to the present, hydrolytic enzyme reactions have been treated as though they proceeded completely in one direction or, more accurately, as though the opposite synthetical reaction were so inconsiderable as to be negligible. We know that the hydrolysis of cane-sugar by acids—the classic reaction of chemical dynamics—is practically complete under ordinary conditions and we should at first expect the same to occur also with enzymic hydrolyses. But recent researches of Y. Osaka (Journ.

Coll. of Science, Tokyo, 1908, **25**, 1) have shown that, even with cane-sugar, if sufficiently concentrated solutions are employed, the equilibrium is apparent just as it usually is with the esters of organic acids. The change which such a reversible system undergoes with lapse of time is the difference between two opposite actions.

For instance, the velocity of hydrolysis of the ester, v_1 is given by the equation:

$$v_1 = k_1 \text{ [ester]},$$

if, as usual, we indicate the concentration by [ ]. The velocity of formation of the ester v_2 is expressed by:

$$v_2 = k_2\text{[acid] [alcohol]},$$

and the actual resultant velocity in a system not in equilibrium and containing ester, acid, alcohol and water, is:

$$v = v_1 - v_2 = k_1\text{[ester]} - k_2\text{[acid] [alcohol]}$$

or, if we start from a pure ester solution of the concentration a and indicate the amount changed in time t by x:

$$\frac{dx}{dt} = v = v_1 - v_2 = k_1(a-x) - k_2x^2. \quad . \quad . \quad . \quad . \quad (21)$$

The ratio between the two velocity constants is, as was shown by van't Hoff (compare Chapter VI), the constant of chemical equilibrium, so that

$$\frac{k_1}{k_2} = K = \frac{\text{[ester]}}{\text{[acid][alcohol]}}.$$

When the constant of the " reverse reaction " is not vanishingly small, the time course of the total reaction v, as can be easily seen, is changed if the product of the reaction x is previously added to the system, and, in general, the progress of a reversible reaction must be retarded by addition of the products of the reaction.

When k_1 and k_2 have been experimentally determined, it is easy to calculate how v changes with increasing additions of x.

For dilute solutions and inorganic catalysts these relations have been completely worked out.[1]

With enzyme reactions another circumstance, which has to be considered, complicates matters to some extent. We have already discussed the assumption, now generally accepted, that the enzymes form compounds with the substrate and with the products of reaction. In no case do we know in what measure an enzyme enters into such combination, but we assume that these compounds exert considerable influence on the time course, so that the velocity of an enzymichydrolysis is altered by addition of the products of the reaction to the system, not only according to Eq. (21), but also by combination (inactivation) of the catalyst.

This influence has not only been observed qualitatively, but has, in many cases, been measured.

Of the experiments showing the retarding influence of the hydrolytic products, that quoted by W. Kühne (Lehrbuch der physiol. Chem., 1866, p. 39) is one of the earliest: If a digestion solution, filtered from excess of undigested fibrin, is placed in a dialyser, most of the peptones diffuse into water, whilst the pepsin remains behind. When the pepsin solution is restored to its original volume by evaporation and to its initial acidity, it dissolves almost exactly as much fibrin as was previously dissolved. It is consequently the peptones which hinder the digestion.

We may also recall the investigation, already mentioned, of Tammann (H., 1891, **16**, 271) which showed in a convincing manner that the products of hydrolysis generally influence the completeness of enzymic reactions; but, as he says, "not only by removal and destruction of the decomposition products can an enzyme reaction be rendered complete, as the same end is attained by repeated additions of enzyme."

Of Tammann's numerous experiments, the following may be mentioned:

To different solutions, each containing 0·51 grm. of amygdalin, were added equal amounts of emulsin and varying quantities of saturated benzaldehyde solution, all the solutions being then

[1] In a modification of this equation devised for reversible enzyme reactions, B. Moore (1906) makes an attempt to take account of the activity of the enzyme.

made up to 25 c.c. When the final state was reached (at 20°), the following quantities of amygdalin were decomposed:

Volume of aldehyde solution.	Percentage of amygdalin decomposed.	
0 c.c.	20·3	
1 c.c.	18·8	
5 c.c.	14·7	
10 c.c.	11·3	Precipitation
Solution saturated with benzaldehyde.	5·7	of emulsin.

Hydrocyanic acid has a still more marked action:

25 c.c. of solution at 30° contained 0·5 grm. emulsin and 0·001 grm.-mol. of amygdalin:

Hydrocyanic acid added.	Amygdalin decomposed.
0·0000 grm.-mol.	23·7
0·0001 "	18·7
0·0002 "	16·4
0·0003 "	12·1

The effect of the third product of the decomposition, glucose, is much less marked, as is shown by the following table, due to Auld:

Minutes.	Grm. of Glucose added.	Amygdalin hydrolysed.
30	0·0	13·5%
30	0·2	13·3
30	0·75	11·8
30	1·0	11·6

In the further investigation of this influence it should be remembered that emulsin consists of several specifically-acting enzymes.

That the diastatic hydrolysis of starch gives an end point which is influenced by the sugar formed, is pointed out by Moritz and Glendinning (Journ. Chem. Soc., 1892, **61**, 689).

Henri has made numerous experiments on the effect of added glucose and fructose on the inversion of cane-sugar, the establishment of an effect of this kind being of importance for the development of his formula. As has been often pointed out, it is greatly to be regretted that his experimental numbers are considerably distorted owing to the mutarotation of glucose, so that quantitatively they are almost valueless. It does appear,

however, that invert-sugar lessens the velocity of inversion. The following table is compiled from Henri's numbers (loc. cit., p. 202):

2 c.c. of enzyme solution+50 c.c. 0·2-normal cane-sugar solution.

t	Without addition. $\frac{x}{a}$	+0·3-normal invert-sugar. $\frac{x}{a}$
99	0·138	0·072
215	0·301	0·160
299	0·407	0·224
459	0·594	0·340
586	0·700	0·417
1202	0·927	0·672

The 0·2-normal cane-sugar solution which is also 0·3-normal with respect to invert-sugar was therefore hydrolysed only half as rapidly as that without invert-sugar.

This retarding influence of invert-sugar seems to be due exclusively to the fructose, as is shown by the following values of $\frac{x}{a}$ given by Henri.

Time.	0·2 N-cane-sugar.	0·2 N-cane-sugar. +0·2 N-glucose.	0·2 N-cane-sugar +0·2 N-fructose.	0·2 N-cane-sugar. +0·2 N-invert-sugar.
75	0·142	0·144	0·123	0·119
184	0·385	0·362	0·317	0·306
275	0·564	0·532	0·457	0·446
445	0·798	0·746	0·672	0·648
605	0·906	0·878	0·799	0·794

As will be seen, the retardation of a reaction by the hydrolytic products is decidedly specific. That this is the case was also brought out very clearly in a table given by E. F. Armstrong (Proc. Roy. Soc., 1904, **73**, 516) to show the effect of the hexoses in delaying the hydrolysis of sugars. Still more striking examples of the specificity of such retardations will doubtless be obtained from the investigation of the polypeptides which has recently been taken in hand.

Kühne's experiment on the influence of the decomposition products on the peptic digestion of fibrin has already been quoted. Bayliss has made experiments on the retardation of the tryptic digestion of casein by albumoses, peptones and amino-acids (Arch. Sci. Biol. St. Petersburg, 1904, **11**, Supplement, pp. 261 et seq.), his results showing that the amino-acids —glycine and leucine were examined—are the most active in this respect.

In investigating the hydrolysis of dipeptides (glycylglycine) by erepsin Euler also made experiments on the influence of the amino-acids (glycine) (H., 1907, **51**, 213). In this case it was found that addition of glycine has only a subordinate effect.

0·10 N-glycylglycine; 0·04 N-NaOH.			0·05 N-glycylglycine; 0·10 N-glycine; 0·04 N-NaOH.		
Minutes.	$1000(a-x)$.	$1000k$.	Minutes.	$1000(a-x)$.	$1000k$.
0	955	—	0	480	—
8	852	6·25	10	414	6·40
16	766	6·00	18	372	6·20
25	678	5·95	27	329	6·08

0·20 N-glycylglycine; 0·05 N-NaOH.			0·10 N-glycylglycine; 0·2 N-glycine; 0·05 N-NaOH.		
0	1860	—	0	900	—
6	1692	6·9	6	829	5·9
12	1545	6·7	12	767	5·8
20	1376	6·55			
30	1210	6·2			

It is here, of course, essential that the relation between the NaOH and the acid present (dipeptide+amino-acid) is not appreciably changed by the addition of glycine.

Special mention must further be made of the work of Abderhalden and Gigon (H., 1907, **53**, 251) on the hydrolysis of glycyl-*l*-tyrosine. In this case the tyrosine in the solution produces a considerable retardation. Similar effects are produced by the previous addition of active amino-acids, especially of those occurring in nature: *d*-alanine, *l*-serine, *l*-leucine, *d*-glutaminic acid, *l*-phenylalanine, *d*-tryptophane and *l*-diamino-trihydroxydodecanic acid.

The order of magnitude of the retardations is shown by the following results:

0·1 grm. glycyl-*l*-tyrosine+ 1 c.c. pressed yeast-juice.		0·1 grm. glycyl-*l*-tyrosine+1 c c. pressed yeast-juice +0·05 grm. *d*-glutaminic acid.		
Minutes.	Rotation.	Minutes.	Rotation.	Rotation corrected for that of the glutaminic acid.
0	+0·70°	0	+0·70°	+0·55°
9	+0·51	7	+0·55	+0·47
23	+0·20	19	+0·54	+0·46
34	+0·00	29	+0·54	+0·46
		49	+0·53	+0·45
		79	+0·53	+0·45
		105	+0·47	+0·39

On the other hand, glycine, *l*-alanine and *d*-leucine exert no retarding action, whilst with the racemic compounds, such as *dl*-alanine, the effects are small. From the specific influences which the hydrolytic products thus show towards the digestion of polypeptides, it must again be concluded that the hydrolysing enzyme enters into direct union with these protein decomposition products. At about the same time C h o d a t (Arch. Sci. phys. nat., 1907, **26**, 112) made similar measurements with the anhydrides of *l*-tyrosine and glycyl-*l*-tyrosine. In extremely high dilutions, the amino-acids have an a c c e l e r a t i n g action. A b d e r h a l d e n and G i g o n also point out the difference between digestion i n v i t r o, where the decomposition products of the proteins have a considerable retarding influence, and digestion in the intestinal canal, where these inhibiting products are rapidly removed by resorption.

In other words: In the organism we have (in certain periods) so-called stationary states, in which the substance, acted on by the enzyme and then removed from the sphere of action of the latter by diffusion or some other method, is continually replaced by an equivalent quantity of fresh starting material. Such cases of stationary chemical processes, for example, with the unimolecular reaction $A \rightarrow B+C$, can be represented by the following scheme.

Entry of A: $\rightarrow$ x grm.-mols. per unit of time.	limiting surface.	Field of reaction: conversion of x grm.-mols. per unit time.	limiting surface.	Exit of B and C: x grm.-mols. of $\rightarrow$ each per unit time.

For the given conditions, we have the simple relation:

$$\frac{dx}{dt} = \text{const.}$$

For such a stationary condition to obtain, it is by no means necessary that a system should be limited by solid walls. H. Goldschmidt (Zeitschr. f. physikal. Chem., 1899, **31**, 235) has studied a chemical process in which the above equation is realised.

If an excess of an ester, slightly soluble in water, is shaken with a dilute hydrochloric acid solution, in which the ester (in so far as it is dissolved) is hydrolysed with a certain velocity, the concentration of the dissolved ester is kept constant merely by the shaking, since the amounts destroyed by hydrolysis are continually removed by the diffusion of the solution. If the velocity of reaction is not very great, the ester disappearing from the aqueous phase owing to the reaction, can be carried away completely by diffusion, so that the concentration of the ester in the aqueous solution is maintained at a constant value, namely, that corresponding with saturation.

The usual equation for a unimolecular reaction,

$$\frac{dx}{dt} = (a-x)k$$

becomes

$$\frac{dx}{dt} = k'',$$

since

$$a-x = a = \text{const.}$$

A condition for such a reaction is, therefore, that the phase in which the reaction occurs, is always saturated with reference to the substrate; this condition may be fulfilled by vigorous shaking of the heterogeneous system, or by employing the body to be dissolved in an extremely finely divided state, so that its surface of contact with the solution is very great. Excessively large surfaces of this kind occur particularly in so-called colloidal solutions.

In this connection, it must be again pointed out that the acceleration or retardation suffered by an enzyme reaction is by no means always to be attributed to combination of the enzyme with the substrate. In many, perhaps even in most, cases it is a question of the alteration of the concentration of the activators (co-enzyme, etc.), either by the reaction products and the substrate acting in a reversible manner on these substances or by the latter being changed or destroyed by secondary reactions. The opportunity has already been taken of pointing out that the deviation from the theoretical course of the reaction—which is termed Schütz's rule—might, according to the data as yet to hand concerning peptic digestion, very well be caused by the gradual fixing of the hydrochloric acid by the albumoses and peptones formed, the acid being withdrawn from both the substrate and the pepsin. It is to be hoped that digestion experiments carried out in vitro and with a constant concentration of hydrochloric acid, may decide this not unimportant question.

According to theory, all reactions, even if they apparently proceed to completion, are finally arrested at a position of equilibrium. Chemical reactions often go so far in one direction that, under ordinary conditions, the equilibrium cannot be detected analytically; only in very concentrated solutions is the equilibrium apparent. The manner in which the equilibrium of a reversible reaction $A \rightleftarrows 2B$ depends on the velocities of the two opposed processes $A \rightarrow 2B$ and $A \leftarrow 2B$, and the way in which natural equilibria and the final states of enzymic reactions are connected, will be described in Chapter VI.

This short résumé of the theory of enzymic dynamics may now be brought to an end. It will be seen later how the experimental data agree with the requirements of theory.

II. EXPERIMENTAL DATA ON THE COURSE OF ENZYME REACTIONS

ESTERASES AND LIPASES

With esterases, that is, with enzymes which hydrolyse lower esters but not neutral fats, Kastle and Loevenhart (Amer. Chem. Journ., 1900, **24**, 491) and Kastle, Johnston and Elvove (ibid, 1904, **31**, 521) have carried out a number of experiments; they used turbid aqueous extracts of pig's liver and pancreas filtered through cloth.

Tubes containing 4 c.c. water, 0·1 c.c. toluene, and 0·26 c.c. ethyl butyrate were heated for 5 minutes at 40°. One c.c. of a 10% extract was then added and after a definite time the solution titrated with N/20-KOH solution:

Minutes.	x	$k.10^5$	$\kappa.E$
5	(6·53)	1354	(0·45)
10	(8·66)	907	(0·40)
15	8·53	597	0·26
20	9·54	500	0·24
25	10·67	500	0·24
30	(9·41)	329	(0·16)
60	17·32	316	0·28
120	25·35	244	0·32
180	28·36	184	0·28

The constant k, calculated from the equation of a reaction of the first order, diminishes regularly and very considerably. Also interpolation shows that the errors of observation, presumably owing to the difficulty of pipetting small volumes of the ester exactly, were very large; the values given in brackets fall quite away from the curve. For the other numbers, the values of κE calculated from formula (18)

$$a\ ln\frac{a}{a-x}-x=\kappa Et,$$

show satisfactory agreement. This formula was deduced (pp. 133–135) on the assumption that the concentration of the free enzyme is inversely proportional to that of the products of the reaction. The same formula is obtained on the supposition—highly probable in this case at least—that the activity and not the concentration of the enzyme is inversely proportional to the reaction products, chief among these being the acid. As was found by Kastle and Loevenhart, the lipase of the pancreas is very sensitive to acids. It would doubtless be of value to ascertain if the formula for unimolecular reactions does not hold with good approximation if a certain quantity of strong acid were previously added in order to depress the action of the products of the reaction.

With a moderate degree of accuracy the above figures and those given below agree with the formula $x/\sqrt{t}=k$.

The measurements made by Stade on an emulsion of egg-yolk and neutralised gastric juice (Hofm. Beitr., 1902, **3**, 291) have been calculated according to the above formula by Arrhenius and found to agree closely with it, as is seen from the following table:

Hours.	x (observed).	x (calculated).	
2	0·204	0·186	$\kappa E=10$
4	0·256	0·257	
6	0·298	0·308	
8	0·353	0·348	
10	0·376	0·383	
25	0·495	0·552	
29	0·515	0·582	
31	0·554	0·596	
35	0·609	0·620	
75	0·775	0·784	

Further, Engel (Hofm. Beitr., 1905, **7**, 77), in a careful investigation with emulsion of egg-yolk and pancreatin on the lines of Volhard's and Stade's experiments, arrived at the result that Schütz's rule holds for the enzymic saponification of fats; that is, for a constant period of digestion, the amounts of digestion are in the ratio of the square roots of the quantities of enzyme, and for the same quantity of enzyme the products

of digestion are proportional to the square roots of the times of digestion. Hence, for one and the same juice, the equation

$$x = k\sqrt{Et},$$

must hold. This is found to be the case.

Pancreatin.	4 hours.			9 hours.			25 hours.			
	x (obs.)	x (calc.)	$\frac{x}{\sqrt{Et}}$	x (obs.)	x (calc.)	$\frac{x}{\sqrt{Et}}$	x (obs.)	x (calc.)	$\frac{x}{\sqrt{Et}}$	
0·04 grm.	17·6	16·8	4·4	18·4	24·5	3·1	35·0	38·3	3·5	Temp. 41°
0·09 "	20·9	24·5	3·5	36·3	35·0	4·0	58·2	53·0	3·8	
0·16 "	35·2	31·6	4·4	48·4	44·6	4·0	72·1	65·0	3·6	

The values of x (calc.) have been obtained by means of Arrhenius's formula, the constant κ being taken as 1. Although the experimental errors are considerable, it is clear that the numbers follow Schütz's rule.

The experimental data obtained by Zellner (Monatsh. f. Chemie, 1905, **26**, 727) with lipase from fly agaric (Amanita muscaria) have been subjected to calculation by Kanitz, who found that the results vary, as in two series of experiments the quotient $\frac{x}{t}$ was constant and in another series the expression

$$\frac{x}{\sqrt{t}}.$$

Euler (Hofm. Beitr., 1905, **7**, 1) hydrolysed ethyl butyrate in aqueous solution with esterases (from the fatty tissues of the pig) and found the law for unimolecular reactions to hold.

Minutes. t	Vol. of baryta solution used in the titration (c.c.). x	Vol. of baryta solution not used. $a-x$	Unimolecular reaction constant. $k.10^4$
0	0	2·70	—
2	0·3	2·40	256
6	0·75	1·95	235
9	1·05	1·65	237
16	1·65	1·05	250

From his observations on the lipase of Lactarius sanguifluus, Rouge (Centralbl. f. Bakt., 1907, **II**,

18, 403, 587) also drew the conclusion that, in dilute solutions, the action of the enzyme is directly proportional to its amount.

Experiments with the true lipases all relate to systems with limiting surfaces perceptible macroscopically (suspensions), since most of the lipases are insoluble in water. Glycerine extracts showing lipolytic activity can be obtained from the pancreatic glands of the pig (A. K a n i t z , H., 1905, **46**, 482). This author followed the course of hydrolysis of olive and castor oils.

Into each of a series of test-tubes were placed 10 c.c. of olive oil, 3·9 c.c. 0·1N-sodium hydroxide, 0·25 c.c.N-calcium chloride, and 1 c.c. of lipase extract, the contents of the tubes being titrated after t minutes. The numbers of c.c. of 0·1N-sodium hydroxide used are given under x.

t	x	$\frac{x}{t}$	$\frac{x}{\sqrt{t}}$
0	0·0	—	—
70	9·2	0·131	1·10
140	12·3	0·088	1·04
288	19·0	0·065	1·12
405	23·1	0·057	1·15
1455	34·2	0·023	0·90

The time-law of the vegetable lipases was first investigated by C o n n s t e i n , H o y e r and W a r t e n b e r g (Chem. Ber., 1903, **35**, 3988), who established the essential fact that considerable quantities of free acid are necessary for the lipases to exhibit their action. If, as suggested by S i g m u n d (Monatsh. f. Chemie, 1890, **11**, 272), powdered castor-oil seeds are ground with water and left for 24 hours at about 40°, small quantities of acid are detectable by titration. Later, however, the quantity of acid formed rises suddenly. This " jump " occurs after 2–3 days at 35–40° or after 4–6 days at 15–20°.

For instance, 5 grms. of castor-oil seeds were pounded with 5 grms. of 1% chloral hydrate solution and the mass kept at 16°.

	Immediately.	After 2 days.	4 days.	6 days.	8 days.
Per cent of ricinoleic acid found	1	3	52	59	59

This is a case of autocatalysis, in which one of the products of the reaction exercises an accelerating action. As to the most

favourable concentration of acid, information is given by the following results, obtained with sulphuric acid:

Normality of the acid	0·02	0·05	0·10	0·12	0·2	0·5
Percentage hydrolysed in 18 hours	25	80	86	84	86	13

In addition to the seed-lipases, only pepsin acts in such strongly acid solutions. According to the authors named above, the idea that the acid transforms a pro-enzyme (zymogen) present in the seeds into an active enzyme, is untenable, since when the seeds are treated for a long time with acid and the latter then removed, they exhibit no alteration; in fact they show as little fat-splitting action in neutral solution as before treatment, and they also become active in presence of acid.

In their examination of the influence of various organic acids on Ricinus-lipase, H. E. Armstrong and Ormerod (Proc. Roy. Soc., 1906, **78**, 378) obtained the following numbers:

Concentration of the acid.	0·01 N.	0·02 N.	0·10 N.	0·50 N.	$k.\ 10^5$.
Acetic acid	5·45	14·9	14·6	13·6	1·8
Succinic acid	2·80	14·6	15·4	12·2	6
Citric acid	7·25	15·3	14·7	1·1	82
Tartaric acid	6·95	15·4	14·2	—	97

Here also there occurs a rather flat maximum. No relation is evident between the strength of the acid—the dissociation constants are given in the last column—and the extent to which the reaction is accelerated or the enzyme activated.

The nature of the added acid seems, indeed, to have but slight influence, and this suggests the idea that the action consists of a liberation of the true activator, itself presumably a weak acid.

In order to ascertain the influence of the quantity of the enzyme on the velocity of hydrolysis, quantities of 0·5 grm. of the Ricinus-seeds were mixed well with 5, 10, 15, 20, 25, and 50 grms. respectively of castor oil and with similar amounts of 2% acetic acid. Assuming that the active mass of the lipase is proportional to the total quantity, and calculating the results obtained after a certain time by means of the formula previously deduced (p. 136):

$$1000\ ln\ \frac{1000}{1000-x} - x = xEt,$$

the following numbers are obtained, these agreeing satisfactorily with the observed values excepting in the case of the largest proportions of enzyme:

Action of 0·5 grm. castor-oil seeds on		After 1 day,		After 2 days,	
grms. of oil.	grms. acetic acid solution.	x (obs.)	x (calc.)	x (obs.)	x (calc.)
50	50	49	49	49	59
25	25	60	65	74	74
20	20	71	69	80	78
15	15	77	75	87	84
10	10	81	83	86	91
5	5	89	94	92	98
		$\kappa = 186$		$\kappa = 300$	

The time-course of the reaction is shown by a number of the experiments carried out by these authors, among them Nos. 26, 28, 38, and 46. The last two were carried out by pounding 5 grms. of castor-oil seeds with 6·5 grms. of castor-oil and either 4 grms. of 0·1 N-sulphuric acid (No. 46) or 4 grms. of acetic acid (No. 38). The results were as follows:

Experiment 46.			Experiment 38.					
0·10 N-sulphuric acid.			0·10 N-acetic acid.			0·40 N-acetic acid.		
t (mins.)	x (obs.)	x (calc.)	t (hours)	x (obs.)	x (calc.)	t (hours)	x (obs.)	x (calc.)
15	12	20	1	52	48·6	1	65	63·9
30	20	27	2	65	62·8	2	86	78·8
45	30	32	3	70	71·5	3	84	86·5
60	33	36	4	72	77·6	4	84	91·2
90	41	43	24	80	99·5	24	91	99·9
150	54	53		$\kappa E = 180$			$\kappa E = 380$	
210	59	59						
330	68	69						
1620	81	97						
	$\kappa E = 1·47$							

With the longer times, the deviations of the observed numbers from the calculated ones are considerable and on this account Arrhenius supposes equilibrium to be set up in these cases. Nicloux (Soc. Biol., 1902, **54**, 840) has also made a thorough investigation of Ricinus-lipase or, as he terms it, the lipolytic action of the cytoplasm of Ricinus-seeds. Nicloux like-

wise found his preparation to be insoluble in water; he emulsified the cytoplasm in the oil—mostly cottonseed oil—to be hydrolysed and added dilute acetic acid. The following numbers were obtained at 18°:

t (minutes)	30	45	60	90	127	150	210	450
Percent. hydrolysed	23·6	33·1	40·4	54·8	67·0	73·2	85·5	94·4
$\kappa E = 1000 ln \frac{1000}{1000-x} - x$	1·10	1·58	1·89	2·73	3·45	3·89	5·12	4·31
$k = \frac{100}{t} \log \frac{100}{100-x}$	0·388	0·387	0·375	0·382	0·392	0·381	0·399	0.278

It is evident that the observed numbers do not follow Schütz's rule; on the contrary, the course of the hydrolysis agrees moderately well with the formula for unimolecular reactions.

A thorough series of experiments with Ricinus-lipase has recently been carried out by Jalander (Biochem. Z., 1911, **36**, 435). For short times (up to 60 minutes) be found proportionality with enzyme-concentrations of 1–4 per 1000. For more protracted action this proportionality disappears, Schütz's rule, $x:\sqrt{E} =$ const. then holding approximately.

Bodenstein and Dietz (Zeitschr. f. Elektrochem., 1906, **12**, 605; Dietz, H., 1907, **52**, 279) have also studied a heterogeneous system. Pancreatic lipase, in the shape of shavings of the tissue of the pancreatic glands of the pig, was emulsified with amyl alcohol containing water and butyric acid or amyl butyrate in solution.

One would expect the velocity equation,

$$\frac{dx}{dt} = k_1 \cdot C_{\text{acid}} \cdot C_{\text{alcohol}} - k_2 \cdot C_{\text{ester}} \cdot C_{\text{water}}$$

to be fulfilled, all the concentrations relating to the enzyme phase. The concentrations of water and alcohol are approximately constant, since only very small quantities of these disappear during the process. On the other hand, the substances must be divided between enzyme and liquid according to Nernst's law of distribution, i.e., $C_{\text{enzyme}} = \alpha . C_{\text{solution}}$. The proportionality factors, like the constant concentrations of alcohol and water, become included in the constants, so that

$$\frac{dx}{dt} = k_1 \cdot C_{\text{acid}} - k_2 \cdot C_{\text{ester}} = k_1(a-x) - k_2 x.$$

If but little water is dissolved in the amyl alcohol, the reverse reaction is very slight and the process goes on according to the simple equation:

$$\frac{dx}{dt}=k(a-x),$$

as is shown by the following table:

$a=0\cdot10$ normal

4% H_2O.			2% H_2O.		
t (hours).	Titre of 5 c.c.	k	t (hours).	Titre of 5 c.c.	k
0·00	13·55	—	0·00	13·55	—
2·00	12·75	0·013	2·50	13·15	0·0064
5·53	11·90	0·010	9·57	11·80	0·0062
10·33	10·35	0·012	14·35	10·95	0·0064
15·17	9·15	0·012	24·45	9·45	0·0063
25·20	7·05	0·012	31·88	8·45	0·0063
32·58	5·85	0·012	47·89	6·55	0·0065
48·83	4·25	0·012	55·27	5·90	0·0064
55·92	3·90	0·011	76·67	4·60	0·0060
77·73	3·00	0·010	100·42	3·25	0·0062
308·00	1·50	—	308·00	1·10	—

On passing to higher contents of water, a final condition attainable from either side is set up and the velocities of the two opposed reactions become measureable. Such a case is presented by the following tables.

Formation of ester.			Hydrolysis of ester.		
t (hours).	Titre of 5 c.c.	k_1	t (hours).	Titre of 5 c.c.	k_2
0·00	13·40	—	0·00	0·00	—
1·98	12·60	0·014	3·63	0·75	0·0075
4·00	11·82	0·014	7·60	1·45	0·0077
6·98	10·80	0·014	16·77	2·35	0·0064
11·55	9·10	0·016	24·05	2·95	0·0070
14·98	8·40	0·016	89·30	4·20	—
25·10	6·55	0·016			
96·95	4·25	—			
		Mean 0·015			Mean 0·0072

Both constants are approximately doubled if the quantity of enzyme is doubled:

Quantity of enzyme.	Constants. k_1	k_2
1	0·015	0·0072
2	0·028	0·014

A. E. Taylor, who hydrolysed triacetin, the acetic ester of glycerine, with powdered Ricinus-seeds, found the course of the reaction to be as with unimolecular reactions. He gives the following results for experiments in which 0·5, 1, and 2% solutions of triacetin were employed. The constants k refer to 18°.

	t (hours).	4	8	16	24	28	32	40	48
0·5%	x (obs.)	0·096	0·162	0·287	0·418	0·489	0·477	0·623	0·652
	k	109	96	92	98	104	88	106	96
1·0	x (obs.)	0·083	0·174	0·338	0·418	0·488	0·542	0·609	0·655
	k	94	104	112	98	104	106	102	96
2·0	x (obs.)	0·098	0·174	0·323	0·431	0·502	0·485	0·595	0·636
	k	112	104	106	102	108	90	98	91

How it happens that in all these experiments the quantity of fat hydrolysed can be calculated by means of the very simple law holding for homogeneous systems, is not very easy to understand. With the hydrolysis of fats and of triacetin this is all the more remarkable, since this hydrolysis takes place in three stages, which doubtless occur with different velocities.

New experiments with esterase from pig's liver have been made by G. Peirce (Journ. Amer. Chem. Soc., 1910, **32**, 1517). The principal results are as follows:

(1) In a solution of given volume and given acidity, the time required for the hydrolysis of a definite quantity of ethyl butyrate is inversely proportional to the concentration of the enzyme. Under similar conditions of acidity, each particle of enzyme hydrolyses the same absolute amount of ester per instant, no matter what the concentration of the enzyme.

(2) With a given concentration of enzyme, the time taken to hydrolyse a given amount of ethyl butyrate is dependent on the acid-concentration but not on the ester-concentration, pro-

vided this is above N/200. In other words, for each concentration of acid a given amount of enzyme hydrolyses very nearly the same amount of ethyl butyrate over a wide range of ester-concentration.

(3) This phenomenon can be brought into conformity with the law of mass action by assuming that the enzyme and the ester form an intermediate compound, which, in concentrations of the ester above N/200 contains most of the enzyme.

AMYLASE

The chemical process of the hydrolysis of starch presumably takes place in several phases, in which the dextrins formed as intermediate products are broken down; amylase may consequently be composed of several enzymes.

In experiments with diastase (amylase), the difficulties attending the varying constitution of the enzyme are supplemented by the complication introduced by a non-individual substrate. As has been shown, more especially by Maquenne's investigations, starch consists in reality of two components, namely, 80–85% of amylose and 15–20% of amylopectin. Amylose forms no paste and in solution is coloured an intense blue by iodine; only in solution is it attacked by malt-diastase. There exist a series of condensation products increasingly soluble in water, amylose being the last of these; the lower members more especially are largely contained in "soluble starch." In the saccharification of pure amylose maltose alone is formed. Amylopectin is a gelatinous substance which is insoluble in water and alkalis and gives dextrin as well as maltose (?) on hydrolysis. It is not yet certain, but is probable that this is brought about by a special amylopectinase and not by the amylase. In any case, in quantitative experiments on amylase the purest possible amylose or, at any rate, "soluble starch" should be employed. For the technical determination of diastase, potato starch is treated according to Lintner's method (see Appendix).

As regards the hydrolysis curves, the experiments made by different authors do not agree especially well. The earliest of these experiments, carried out by H. Brown and Glendinning (Journ. Chem. Soc., 1902, **81**, 388) with malt-extract do

not correspond with the simple logarithmic curve. The results are expressed better by the formula

$$\frac{1}{t} \log \frac{a+x}{a-x} = k_H,$$

as is shown by the following numbers.

3% solution of starch with 0·25 c.c. malt-extract per 100 c.c.:

Minutes.	x	$k.10^5$	$k_H.10^5$	Temperature, 51–52°.
10	0·1084	498	·472	
20	0·2250	553	497	
40	0·4350	620	506	
60	0·6150	690	518	
80	0·7385	728	514	
100	0·8150	732	495	
120	0·8800	762	497	
140	0·9220	791	497	
160	0·9500	813	492	
	3% solution of starch with 1 c.c. malt-extract per 100 c.c.			
10	0·081	366	352	Temperature, 21°
20	0·163	386	357	
40	0·308	399	345	
60	0·440	419	341	
70	0·506	437	345	

Henri's experiments (Lois générales etc.), on the other hand, follow the logarithmic law; whether the method employed (determination of the change of reducing power) is free from objection depends on the purity of the amylase used.

Also with saliva-diastase, A. E. Taylor obtained the following values:

Substrate 0·25%								
t (minutes.)	30	45	60	75	90	120	150	180
k ($\times 10^6$)	490	465	455	470	465	455	460	455
Substrate 0·5%								
k ($\times 10^6$)	430	420	390	415	405	395	430	410
Substrate 0·75%								
k ($\times 10^6$)	390	370	385	390	380	370	365	370

As Taylor emphasised, the values of k vary considerably with the concentration of the substrate.

In the course of a valuable investigation on pancreas-amylase, Kendall and Sherman (Journ. Amer. Chem. Soc., 1910,

32, 1087) found: (1) that the initial speed of conversion for a constant amount of enzyme was the same for different concentrations of starch; (2) that the speed of the reaction diminished the more rapidly, the smaller the initial concentration of the starch.

For the formula deduced by these investigators to express the course of the reaction, their original paper must be consulted.

As regards the influence of the concentration of the amylase, Henri found, with a vegetable preparation, proportionality between the concentration of the enzyme and the quantity of starch hydrolysed per unit of time.

Taylor arrived at the same result with saliva-diastase. Brown and Glendinning (Journ. Chem. Soc., 1902, **81**, 381), however, found the velocity of reaction to be proportional to the square-root of the concentration of the saliva-diastase, and Klempin (Biochem. Z., 1908, **10**, 206), from experiments with oat-diastase, concluded that Schütz's rule, $E\sqrt{t}=\kappa$, holds for this enzyme.

Pawlow's experiments (Arbeit der Verdauungsdrüsen, Wiesbaden, 1898), which were carried out with Mett's capillary tubes and are of interest in themselves, hardly give any information concerning the course of the saccharification of starch, since they dealt with the liquefaction of starch-paste and since also the velocity of diffusion exerts a determining influence on the velocity of the process.

A large number of experiments were made by Mdlle. Philoche (Journ. de Chim. physique, 1908, **6**, 213, 355) with "Merck" diastase and taka-diastase, starch and glycogen being used as substrates.

The constants of the formula for unimolecular reactions diminish rapidly as the reaction proceeds. The following numbers were obtained with 1% starch solution, the concentration of the diastase being 1: 20,000.

Minutes.	$\frac{x}{a}$.	$10^5\, k=\frac{1}{t} ln \frac{a}{a-x}$.
21	0·06	123
51	0·11	100
113	0·15	62
224	0·26	58
390	0·36	50

The influence of the concentration of the starch on the amount of maltose formed in 60 minutes is shown by the following table:

Concentration of starch (%).	Quantity of maltose (60 mins.).
1	0·24
1·5	0·30
2	0·338
2·5	0·397
3	0·397

Activators and Inhibitors. The following substances favour the hydrolysis of starch: vanadium and aluminium salts, ammonium and calcium phosphates, asparagine, amino-acids, proteins and picric acid (Effront, Enzymes and their Applications, p. 117; Soc. Biol., 1904, **57**, 234; Allg. Brauer- und Hopfenzeitung, 1905, **45**). The accelerations are sometimes very considerable; thus an addition of 0·05 grm. of asparagine to 100 c.c. of a starch solution containing amylase increases the velocity sevenfold. Carbon dioxide also has an accelerating action, especially when under increased pressure (Detmer, Müller-Thurgau).

The optimum activity of vegetable diastases occurs with a slight excess of hydrogen-ions (small quantities of organic acids); hydroxyl-ions, even in very small concentration, retard the hydrolysis, but the inactivation is annulled immediately the alkali is neutralised. The optimum temperature (measured by Kjeldahl by the reducing power of the hydrolytic products) is 63°.

INVERTASE

On the hydrolysis of cane-sugar by invertase numerous quantitative investigations have been carried out. Of these, besides the earlier work of Kjeldahl (Medd. fra. Carlsberg Lab., 1881), mention must first be made of the researches of Tammann (Zeitschr. f. physikal. Chem., 1889, **3**, 25) and of those executed almost simultaneously by O'Sullivan and Tompson (Journ. Chem. Soc., 1890, **57**, 834). These investigators showed first that the reaction is a catalytic one and O'Sullivan and Tompson found it to be unimolecular. This result was contested later by Duclaux (Traité de Microbiologie, Vol. 2, 129), while Henri (Zeitschr. f. physikal. Chem., 1902, **39**, 194) also arrived at a formula differing from the unimolecular one, namely, the expression (compare p. 129),

$$2k_H = \frac{1}{t} \cdot \log \frac{a+x}{a-x},$$

which corresponded moderately well with his experimental data. Still later Bodenstein deduced the complicated formula mentioned on p. 129, and finally Henri (Thèses, p. 92) arrived at the following equation:

$$k_B = \frac{a}{t}\left[(m-n)\frac{x}{a} + \log\frac{a}{a-x}\right] + \frac{1}{t}\log\frac{a}{a-x},$$

which he obtained by integration of the differential Eq. (12) of p. 131. As has been already stated, it has recently been found that the experimental data of Henri and his collaborators are considerably influenced by the mutarotation of glucose and therefore give no definite information as to the time-course of the hydrolysis of cane-sugar; further, in these experiments the concentration of the hydrogen-ions was not defined. Hudson (Journ. Amer. Chem. Soc., 1908, **30**, 1160, 1564) has performed a valuable service not only in demonstrating the reliability of O'Sullivan and Tompson's data, but also in continuing and considerably extending the investigations of these workers.

O'Sullivan and Tompson had rightly recognised that glucose formed from cane-sugar by inversion appears firstly in a mutarotating condition and that therefore the optical activity of a solution which has been inverted by enzyme affords no measure of the progress of the reaction. To the test-portions removed after definite times from their solutions they hence added a little alkali, which annuls the mutarotation almost instantaneously. Their results show that experiments carried out in this way correspond with the unimolecular formula, but that, if the precaution mentioned is not taken, the constant increases considerably. Their numbers are as follows:

Rotation.			$k = \frac{1}{x} ln \frac{a}{a-x}$.	
Minutes.	Without alkali.	With alkali.	Without alkali.	With alkali.
0	74·5°	69·4°	—	—
37	57·9	37·6	0·0021	0·0046
152	0·6	−20·4	0·0037	0·0074
268	−19·1	−24·8	0·0041	0·0058
∞	−27·8	−27·8	—	—

The constancy of the figures in the final column leaves something to be desired and Hudson's numbers may well be given here:

Rotation.			$10^5 k = \frac{10^5}{t} \cdot \log \frac{a}{a-x}$.	
Minutes.	Without alkali.	With alkali.	Without alkali.	With alkali.
0	24·50°	24·50°	—	—
30	16·85	14·27	396	558
60	10·95	7·90	399	530
90	4·75	3·00	464	539
110	1·95	0·80	482	534
130	−0·55	−1·49	511	559
150	−2·20	−2·40	522	533
∞	−7·47	−7·47	—	—

As is seen from this and other tables given by Hudson, the values of k are constant.

According to Sörensen (Biochem. Z., 1909, **21**, 131) and Michaelis and Davidsohn (Biochem. Z., 1911, **35**, 386), however, this constancy of the reaction-coefficient is observed only with a certain concentration of the hydrogen-ions. With different H·-concentrations, Sörensen obtained the following results:

Temperature 52°

$H^{\cdot}=0\cdot2.10^{-6}$		$H^{\cdot}=0\cdot1.10^{-3}$		$H^{\cdot}=0\cdot2.10^{-3}$	
t	k	t	k	t	k
2	—	2	—	2	—
17	91	17	127	17	53·6
32	103	32	127	32	39·3
47	111	47	132	47	26·1
62	127	62	135	62	18·2
92	147	92	149	92	15·3
122	230	122	126	122	11·2

While, therefore, with H·-concentration of $0\cdot2.10^{-6}$, the coefficient k increases, with $H^{\cdot}=0\cdot1.10^{-3}$ it remains constant and with $H^{\cdot}=0\cdot2.10^{-3}$ it diminishes. Michaelis and Davidsohn explain this behaviour on the assumption that

the constancy of the values of k is brought about by the compensating effect of the destruction of the enzyme on the increasing values of the coefficient. Considering the high temperature, 52°, chosen for Sörensen's experiments, this view is most probably accurate, and at lower temperatures, and higher concentrations of hydrogen-ions ($H^{\cdot} = 10^{-3}$) Michaelis and Davidsohn found increasing values for k.

That other experimenters have been unable to obtain agreement with the law of mass action, cannot therefore, be due solely to non-removal of the mutarotation. Thus, H. E. Armstrong and Glover, in comparing the actions of invertase on cane-sugar and on raffinose (Proc. Roy. Soc., 1908, **80**, 312), rendered the sugar solutions alkaline before reading them in the polarimeter, but still obtained no greater constancy, as is shown by the figures in the left-hand part of the following table:

34·2 grms. cane-sugar +4 c.c. invertase-extract per 1000 c.c.			59·4 grms. raffinose +4 c.c. invertase-extract per 1000 c.c.	
Minutes.	Percentage hydrolysed.	$k.10^5$.	Percentage hydrolysed.	$k.10^5$.
0	0·0	—	0·0	—
5	8·3	753	1·8	157
15	25·9	868	5·9	176
25	39·5	865	13·4	249
40	62·4	1062	20·1	243
60	78·2	1102	29·3	251
95	91·1	1106	41·6	246
140	93·7	859	53·4	237
200	95·1	656	66·6	238
260	96·0	537	77·9	252
∞	100·0			

These tables show that invertase hydrolyses cane-sugar into fructose and glucose about four times as rapidly as this enzyme decomposes raffinose into fructose and melibiose.

Our knowledge of the other conditions governing the action of invertase is due to the work of O'Sullivan and Tompson and of Hudson, confirmation of which has been supplied by Taylor (Journ. of Biol. Chem., 1909, **5**, 405). The proportionality found by the first-named authors to exist between the velocity of inversion and the concentration of the enzyme is completely confirmed by Hudson's results.

O'Sullivan and Tompson give the following table (loc. cit., p. 848):

Temp.	Invertase preparation, grms.	N-H_2SO_4.	Time readings.		Reading in 2 d.m. tube.	Minutes taken to reach zero rotation.	
			Beginning.	End.		*A* (obs.).	*B* (calc.).
15·5°	0·15	0·00187	11 40	4 41	− 2·0°	283·0	291
15·5	0·45	0·0031	3 00	4 40	− 1·8	94·8	96·3
15·5	1·50	0·0050	11 56	12 26	+ 1·0	30·7	29·1
56·5	0·0345	0·00025	11 00	12 43	+16·5	157·6	157·1
56·5	0·0722	0·000375	11 22	12 15	+13·5	74·8	75·1

The third column gives the acidity of the solution, the concentrations of acid used having been found by preliminary experiments to be the most favourable to the velocity of reaction. In the last column but one, marked *A*, are given the times elapsing before the rotation falls to 0° and in the last column, *B*, the times which would be necessary for this to occur on the assumption of proportionality between concentration of enzyme and velocity of reaction.

Also with change of the concentration of sugar, the requirements of theory seem to be satisfied within very wide limits by O'Sullivan and Tompson's results, that is, in equal times one and the same quantity of enzyme hydrolyses equal proportions of the sugar, no matter what the concentration of the latter may be.

On the other hand, A. J. Brown (Journ. Chem. Soc., 1902, **81**, 373) gives numbers indicating that a given quantity of enzyme inverts the same absolute amount of sugar in a definite time:

Grms. of cane-sugar per 100 c.c.	Grms. of cane-sugar inverted in 60 minutes.	Percentage of cane-sugar inverted in 60 minutes.
4·89	1·230	25·2
9·85	1·355	13·8
19·91	1·355	6·8
29·96	1·235	4·1
40·02	1·076	2·7

But in dilute sugar solutions containing relatively large amounts of enzyme, the action of a given quantity of enzyme is, according to Brown, proportional to the concentration of the sugar.

Grms. of cane-sugar per 100 c.c.	Grms. of cane-sugar inverted in 60 minutes.	$10^5\,k = \frac{10^5}{t} \log \frac{a}{a-x}$.
(2·0)	(0·308)	(132)
1·0	0·249	219
0·5	0·129	239
0·25	0·060	228

Hudson collects his results in the following table:

Influence of the Concentration of Invertase on the Velocity of Inversion at 30°

Concentration of the invertase, E.	Minutes. t	Product. $E.t.$	Percentage of cane-sugar inverted with the following amounts of cane-sugar per litre.		
			45·5 $\frac{\text{grms.}}{\text{litre}}$	90·9 $\frac{\text{grms.}}{\text{litre}}$	273 $\frac{\text{grms.}}{\text{litre}}$
2·00	15	30	73·2	45·3	11·2
2·00	30	60	93·0	74·2	22·0
1·50	20	30	73·2	44·8	11·2
1·50	40	60	92·8	74·5	22·7
1·00	30	30	72·9	45·3	11·5
1·00	60	60	93·0	74·7	22·3
0·50	60	30	72·9	45·2	11·4
0·50	120	60	92·7	74·5	22·6
0·25	120	30	73·1	45·2	10·9
0·25	240	60	92·7	74·7	21·9

The results are therefore as follows:

1. Proportionality exists between the amount of sugar inverted per unit of time and concentration of the enzyme.

2. The concentration functions are, in addition, dependent on the relative quantities of substrate and enzyme. So long as the enzyme is not present in large excess the relative amount of hydrolysis diminishes as the amount of substrate is increased.

With his erroneous method, Henri observed a relation indicated by the following table. The number of milligrams of sugar inverted after the first minute in a c-normal cane-sugar solution is denoted by n:

n	0·58	1·41	2·40	2·96	4·65	5·04	4·45	2·82	1·15
c	0·01	0·025	0·05	0·10	0·25	0·50	1·00	1·50	2·00

Apart from the fact that Hudson's investigation has led again to a method which is free from objection, the observation

that cane-sugar gives rise first of all to α-glucose possesses on little interest. (Its bearing on the enzymic synthesis of disaccharides will be considered later.) From the work referred to we may take the following extract:

From cane-sugar, not only glucose but also fructose is formed in a labile (characterised by high rotatory power) modification. But the disappearance of the mutarotation—measured at 30°—in the case of fructose (constant of reaction $k=0\cdot186$) proceeds about 11 times as fast as with glucose ($k=0\cdot0167$). Dissolved invertase-preparations have no influence on these velocity constants. Hence, in a cane-sugar solution undergoing enzymic hydrolysis, the difference between the apparent and the actual degree of hydrolysis depends almost entirely on the alteration of the rotation of the glucose. If by means of a very active invertase, a cane-sugar solution can be inverted almost instantaneously, all further alteration in the rotation of the solution must be attributed almost wholly to the mutarotation of the glucose and the velocity of this change must be nearly coincident with the fall of rotation occurring with pure glucose. This has now been actually confirmed.

Use was made of an invertase solution so active that 72% of the cane-sugar was inverted within half a minute. The final rotation was determined after addition of a little sodium hydroxide solution:

Minutes.	Rotation without alkali.	$\frac{10^3}{t}\cdot\log\frac{a}{a-x}$	Minutes.	$\frac{10^3}{t}\cdot\log\frac{a}{a-x}$
0	33·50°	—	—	—
3	11·88	99	—	—
4	7·32	99	—	—
5	4·77	93	—	—
9	− 0·35	72	—	—
10	− 1·35	69	—	—
13	− 3·57	63	0	Commencement
16	− 4·90	57	3	32·3
19	− 6·03	54	6	33·4
23	− 7·15	50	10	33·6
29	− 7·92	44	16	28·7
30	− 8·22	45	17	30·7
∞	−10·22	—	—	—
				31·7

In an equally concentrated solution of invertase the velocity constant of the disappearance of the mutarotation of glucose was found to be $k.10^3 = 29 \cdot 9$, which is in good agreement with the value, $31 \cdot 7$, given above.

Hudson's excellent investigations have been treated at length, because the results obtained with invertase are of importance to the consideration of the enzymic decomposition of other disaccharides and of the glucosides, where the mutarotation of the hexoses makes itself felt to a greater or less extent as a source of error.

Worthy of notice is the influence of the products of the reaction on the course of inversion. As is shown by the experiments of E. F. Armstrong (Proc. Roy. Soc., *B*, 1904, **73**, 500), Barendrecht (Zeitschr. f. physikal. Chem., 1904, **49**, 456) and also Henri (loc. cit.), fructose retards inversion to a much greater extent than glucose does. According to Barendrecht, galactose also exercises a retarding influence, which is, however, less than that of fructose.

The marked acceleration of enzymic inversion by acids has already been mentioned.

It was found by O'Sullivan and Tompson that invertase is very sensitive to small amounts of acid, and this observation has been confirmed by Hudson (loc. cit.). According to the work of the latter, invertase shows its optimum activity in about 0·0006 normal hydrochloric acid. Sörensen, who made a very thorough investigation of the influence of acidity on enzyme actions (Biochem. Z., 1909, **21**, 131) found the optimum concentration of the hydrogen-ions for invertase to be $10^{-4 \cdot 4} - 10^{-4 \cdot 6}$. Very weak acids, like carbonic acid, produce corresponding accelerations.

The slightest excess of OH-ions brings the reaction to a standstill.

The enzyme is almost entirely uninfluenced by antiseptics such as toluene and chloroform.

The temperature-optimum is given as 50–60°. The inactivation constant, $k_t 10^3$, of invertase from a yeast of Frohberg type was found to have the value 4 at 60° and with a H·-concentration of 10^{-6} in aqueous solution (af Ugglas, H., 1910, **65**, 124).

The velocity of inversion by living yeast has been studied by Euler and S. Kullberg (H., 1911, **71**, 24).

In this case the system is macro-heterogeneous, so that the velocity of diffusion should exert an influence on the course of the process.

Here also the inversion corresponds with the formula for unimolecular reactions.

Temperature, 20°.

	Inversion mixture.	Time (mins.).	Rotation.	$A-x$	$k.10^4$.
1*a*	0·25 grm. fresh distillery yeast +20 c.c. 20% cane-sugar solution+5 c.c. H_2O+1 c.c. chloroform; the reaction was stopped by 5 c.c. 0·4N-NaOH solution	0	7·15°	9·44	—
		17	6·25	8·54	26
		25	5·80	8·09	27
		34	5·25	7·54	29
		∞	−2·29	—	—
1*b*	Same as above, but without addition of chloroform	0	7·15	9·44	—
		17	6·20	8·49	27
		25	5·84	8·13	26
		36	5·30	7·59	26
		∞	−2·29	—	—

It will be seen that chloroform exerts no influence on inversion by living yeast. Between 20 and 30°, the effect of the temperature is the same as with inversion by dissolved enzyme.

As is well known, invertase attacks the trisaccharide, raffinose, decomposing the cane-sugar group present. This hydrolysis proceeds more slowly than that of free cane-sugar; it has been investigated by H. E. Armstrong and Glover (Proc. Roy. Soc., 1908, **80**, 317).

MALTASE

As with emulsin, so also with maltase, E. F. Armstrong (Proc. Roy. Soc., 1904, **73**, 508) found the reaction-constants of the first order to diminish considerably.

Maltose, 5%.			Maltose, 10%.		
Hours.	x	$k.10^4$	Hours.	x	$k.10^4$
1	7·3	329	1	4·7	209
2	13·9	325	3	11·7	180
4	24·4	304	5	17·8	170
7·25	31·7	229	23	23·9	52
23	35·2	82	28	25·0	45
			47	31·4	35

In striking contrast to these are the numbers of Henri and Mdlle. Philoche (Soc. Biol., 1904, **57**, 171), of Henri and of Terroine (Archivio di Fisologia, 1904, **2**, 1), who found that the constants k for a unimolecular reaction at first rise, whilst the constants k_H for a definite initial concentration remain comparatively constant.

Terroine (loc. cit., p. 4). Maltose, 4%.			Philoche (loc. cit., p. 6). Maltose, 4%.		
Minutes.	$k.10^5$.	$2k_H.10^5$.	Minutes.	$\frac{x}{a}$	$k.10^5$.
50	88	167	63	0·176	134
112	86	156	120	0·312	135
175	103	170	181	0·441	139
230	127	202	241	0·588	163
349	119	176	363	0·753	167
470	134	184	480	0·824	157
588	122	166	600	0·859	142
780	113	148	750	0·869	117
903	106	148	930	0·900	107

The constants vary, however, with the dilution of the maltase, so that Henri first employed formula (3), p. 129, for the calculation ($m=3$, $n=1$), but as the agreement with the experimental data was not satisfactory, he proposed the introduction of new constants into this formula.

Further, Herzog (Zeitschr. f. allg. Physiol., 1904, **4**, 177) obtained different results, which he calculated according to formula (1*e*), p. 129, with variable coefficients.

Maltose, 3·67%. $\epsilon=1·12$.				Maltose, 3·217%. $\epsilon=2$.			
Minutes.	$\frac{x}{a}$	k	$k_1(1-\epsilon)10^5$.	Minutes.	$\frac{x}{a}$	k	$k_1(1-\epsilon)10^5$.
30	0·128	195	27	20	0·066	149	16
60	0·215	175	23	40	0·138	161	19
120	0·328	144	22	80	0·193	116	15
180	0·415	127	22	120	0·248	103	14
240	0·471	115	20	180	0·312	90	15
370	0·567	76	21	240	0·358	80	15
590	0·631	73	20				
620	0·687	81	22				

It is very probable that the action of maltase, like invertase-action, depends on the concentration of the H·-ions present. It is most desirable that a new investigation should be made in which this influence is considered; more simple laws of reaction would then probably be found to hold.

According to Croft Hill and to Lintner and Kröber (Chem. Ber., 1895, **28**, 1050) the optimum temperature is 40°. Aspergillus-maltase is stated to be only slightly sensitive towards chloroform (Hérissey, Soc. Biol., 1896, **48**, 915). In working with yeast-maltase Fischer recommends the use of toluene.

LACTASE

Quantitative measurements have been made by E. F. Armstrong (Proc. Roy. Soc., 1904, **73**, 506).

In the various series of experiments, the reaction-constants of the first order diminish considerably, but not regularly. As examples, the following tables may be given·

Two Grms. Milk-sugar per 100 c.c.

I. 100 c.c. enzyme-extract.			II. 40 c.c. enzyme-extract.		
Hours.	x	$k.10^4$	Hours.	x	$k.10^4$
1	22·1	1085	0·33	3·2	423
2	31·2	812	0·66	6·4	430
3	38·9	713	1	9·6	438
4	45·8	665	1·5	13·2	410
5	51·5	629	2	16·4	389
6	56·6	664	3	20·8	338
10	69·0	509	5	25·2	252
17	84·2	471	23	47·6	122
23	92·4	461	100	89·6	82

The constants are evidently dependent on the concentration ratio, enzyme : substrate. With a relatively large amount of enzyme, the constant diminishes continuously, but if less enzyme is present, equal amounts of sugar are at first hydrolysed in equal intervals of time.

The following table shows the influence of the concentration of lactase on the velocity of hydrolysis of a 5% milk-sugar solution; the quantities of sugar hydrolysed are given in percentages:

c.c. Lactase.	1·5 hour.	20 hours.	25 hours.	45 hours.	68 hours.
1·0	0·15	2·2	2·6	3·9	4·8
2·5	0·4	5·8	6·8	10·2	12·6
10.	1·6	23·3	—	38·6	48·5
20.	3·2	45·8	54·5	—	—

The quantities hydrolysed are approximately proportional to the enzyme-concentrations, so long as these are not too high. As is shown by the next table, very small amounts of enzyme are able to hydrolyse only small amounts of sugar; their activity then ceases, indicating that the products of hydrolysis, glucose and galactose, combine with the enzyme and so withdraw it from the reaction with the substrate.

5% Milk-sugar solution; amounts hydrolysed in percentages.

c.c. Lactase.	24 hours.	144 hours.
0·66	2·3	2·3
1·0	3·2	3·5
2·0	6·3	7·4
5·0	15·4	34·0

If the quantity of milk-sugar is varied, it is found that, with large proportions of enzyme, the amount hydrolysed in unit time is proportional to the concentration of the sugar, so that the values of k are equal.

Percentages of Milk-sugar Hydrolysed

Milk-sugar per 100 c.c.	Hydrolysed after 3 hours.	$k.10^4$.
1·0 grm.	0·185	296
0·5 "	0·098	298
0·2 "	0·0416	337

For comparatively large amounts of substrate the percentage of sugar hydrolysed is inversely proportional to its concentration, so that in unit time equal absolute amounts of sugar are decomposed, no matter what the concentration.

According to H. E. Armstrong, E. F. Armstrong and Horton, emulsin contains a gluco-lactase. In a series of experiments carried out by Armstrong (Proc. Roy. Soc., 1904, **73**, 507) with milk-sugar and emulsin, the values of k fell rapidly.

2 grms. lactose, 0·2 grm. emulsin } per 100 c.c.				2 grms. lactose, 0·4 grm. emulsin } per 100 c.c.			
Minutes.	x	$k.10^4$.	$\frac{x}{\sqrt{t}}$	Minutes.	x	$k.10^4$.	$\frac{x}{\sqrt{t}}$
0·5	3·2	282	4·5	—	—	—	—
1·0	4·8	214	4·8	1·0	4·9	218	4·9
2·0	6·4	143	4·5	2·0	7·5	169	5·3
3·0	7·6	114	4·4	—	—	—	—
4·5	9·0	91	4·2	4·5	9·4	95	4·4
6·0	10·0	91	4·1	6·0	10·6	81	4·3
23·0	19·7	41	4·1	23·0	30·5	69	2·0
29·0	22·0	37	4·1	29·0	35·0	64	2·0
48·0	29·0	31	4·2	48·0	47·8	59	2·2
53·0	30·7	30	4·2	53·0	50·0	57	2·2
144·0	62·2	29	5·2	144·0	84·0	55	7·0
264·0	77·5	24	4·8				

No simple relation between the time and amount of reaction is at first evident from these figures, and the tables given in this paper indicate none between the velocity of reaction and the concentration of enzyme. The diminution of the constant may be explained by the retarding influence of the products formed by the reaction.

Later investigations (Proc. Roy. Soc., 1908, **80**, 326) show good agreement with a unimolecular reaction, if relatively large quantities of enzyme (almond-extract) are employed.

5% Milk-sugar solution

Hours.	40 c.c. enzyme-solution.		60 c.c. enzyme-solution.	
	x	$k.10^4$.	x	$k.10^4$.
2	15·2	358	18·5	444
3	18·5	296	22·7	373
5	21·1	251	36·5	394
7	33·5	253	44·9	380
9	42·2	264	52·5	360
10	44·0	252	53·5	332
24	73·4	240	76·0	258

As is seen from the third and fifth columns, there is here also no proportionality between concentration of enzyme and velocity. Only relatively small amounts of enzyme have hydrolysing actions proportional to their concentrations, as is shown by the following figures:

Amount of enzyme.....	10	20	40	60 c.c.
Velocity constant, $k.10^4$.....	107	212	279	385

Like invertase and maltase, lactase exhibits its optimal activity in faintly acid solution: according to Bierry and Salazar (C. R., 1904, **139**, 381), with 0·002–0·004% of HCl, which corresponds with a concentration of hydrogen-ions of about 10^{-3}. Lactic acid is stated by Bokorny (Maly's Jahrb., 1903, **33**) to exert a specific accelerating influence.

ENZYMES OF EMULSIN

1. β-Glucosidase

The first quantitative investigation of emulsin was made by Tammann (H., 1891, **16**, 298 et seq.), who examined the action of this enzyme on amygdalin, salicin, arbutin and coniferin. As the following comparison of the calculated and observed values of $(a-x)$ shows, at 25° the process appears to be unimolecular:

Decomposition of Salicin by Emulsin

t (hours).	$a-x$		k
	found.	calc.	
1	87	88	0·061
3	68	67	0·057
5	42	52	0·075
8	35	35	0·058
12	24	21	0·052
26	9	3	0·040

Important progress is marked by the work of Hudson and Paine (Journ. Amer. Chem. Soc., 1909, **31**, 1242) on the decomposition of salicin. These authors paid attention to the facts that the hydrolysis of salicin yields β-glucose and that the reaction is extremely sensitive towards hydrogen and hydroxyl-

ions. The following numbers show that, under suitable experimental conditions, satisfactory constancy of the velocity constant k of the first order is obtained.

Minutes.	Specific rotation (alkaline solution).	10^5k.	
0	−62·0°	—	Temperature, 30°
10	−54·5	360	
20	−48·7	330	Concentration of
30	−41·6	353	salicin,
35	−39·5	339	5%
85	−15·8	374	
145	+ 2·9	350	
∞	+32·2	—	

The influence of acids and bases is indicated by the following table:

Concentration of NaOH.	Activity of the emulsin.	Concentration of HCl.	Activity of the emulsin.
0·005	0	0·00027	222
0·0009	138	0·0005	225
0·0005	195	0·0018	242
0·00009	222	0·005	255
		0·009	206

The optimal activity is hence shown with 0·005 grm.-mols. of HCl per litre.

Auld's experiments (Journ. Chem. Soc., 1908, **93**, 1251) on the hydrolysis of salicin by an enzyme (phaseolunatase) present in Phaseolus lunatus also seem to indicate constancy of the values of k (Table III).

	Hours.	x	k
Hydrolysis of salicin by phaseolunatase at 39·5°	0·5	15·8	141
	1 0	30·9	145
	2·0	59·0	156
	3·0	68·7	147

Herzog (K. Akad. v. Wetensch., Amsterdam, Sitzungsber., 1903, and Zeitschr. f. allg. Physiol., 1904, **4**, 163) has likewise made experiments on the decomposition of salicin by emulsin:

Minutes.	$\frac{x}{a}$	$k.10^5$.	$k_H = \frac{10^4}{t} \log \frac{a-\epsilon x}{a-x}$	Temp. 25°.
24	0·174	346	15	
54	0·354	351	16	Salicin solution,
86	0·450	302	14	0·07 N.
210	0·691	243	13	
270	0·775	239	14	$\epsilon = 0·6$
371	0·847	219	14	

2. Amygdalase and Hydroxynitrilase

The investigations of Armstrong, on the one hand, and Rosenthaler, on the other, indicate that the name amygdalase should be given to that enzyme which hydrolyses amygdalin into mandelonitrile glucoside and glucose. A β-glucosidase present in "emulsin" then decomposes the mandelonitrile glucoside further into glucose and mandelonitrile, and the latter product is finally broken down into benzaldehyde and hydrocyanic acid by the hydroxynitrilase. So that three enzymes take part in the hydrolysis of amygdalin. It can, therefore, hardly be expected that the formation of the final products should correspond with a simple reaction-formula.

The first investigation of the system amygdalin-emulsin was made by Tammann; certain of his experiments on the retardation of the reaction by the products formed have already been referred to in the preceding section (p. 139).

Auld (Journ. Chem. Soc., 1908, **93**, 1251) has recently made a very thorough investigation of the hydrolysis of amygdalin. He followed the reaction by titrating the liberated hydrocyanic acid with iodine and found increasing values for the unimolecular constant:

300 c.c. 2% amygdalin solution + 15 c.c. 2% emulsin solution.

	Minutes.	x	$k.10^5$.
Temperature 40°	10	6·1	255
	80	45·2	295
	100	54·9	309
	150	75·3	347
	1360	93·3	

(x indicates the quantities of amygdalin decomposed, in percentages.)

H. E. and E. F. Armstrong and Horton (Proc. Roy. Soc., 1908, **80**, 330) give their results in the form of the following curves (Fig. 4):

The curve representing the glucose formed is not coincident with that showing the hydrocyanic acid liberated, which would be understandable if these two substances were set free in two different reactions effected by two different enzymes. These authors have therefore done right in not calculating the reaction-

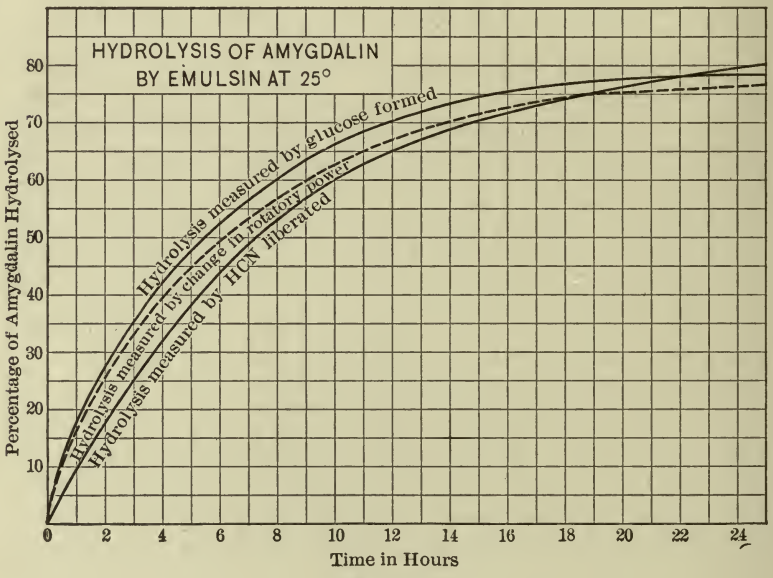


Fig. 4.

constants for the complex of reactions comprised in the hydrolysis of amygdalin.

Similar results are given by Auld (loc. cit., p. 1268) for the temperature 41°.

Quantity of enzyme.....	2	3	4	6	12	25	50 c.c.
Percent. hydrolysis in 10 mins.	3·7	6·05	8·7	12·5	17·2	21·6	24·1

If dilute amygdalin solutions are employed, the constants are, as they should be theoretically, independent of the concentration of amygdalin (Auld, loc. cit., p. 1270).

Hydrocyanic acid and glucose produce retarding effects.

Mention must finally be made of Henri and Lalou's polarimetric experiments (Soc. Biol., 1903, **55**, 868) on the simultaneous decomposition of amygdalin and salicin.

t minutes.	2% salicin.	2·5% amygdalin.	2% salicin +2·5% amygdalin	4% salicin.	1·25% amygdalin.
46	0·67	0·97	1·05	1·08	0·90
130	1·58	2·38	3·63	2·25	1·57
268	2·32	3·15	4·22	3·45	1·56
∞	3·15	3·17	6·32	6·30	1·59

It will be seen that the hydrolysis of the mixture takes place much more slowly than that of the two constituents separately. This fact also indicates combination of enzyme and substrate.

The enzyme is moderately resistant to chloroform and toluene and its optimum temperature is given as 45°.

PROTEOLYTIC ENZYMES

The first accurate experiments on the time-course of the decomposition of protein by pepsin are due to E. Schütz (H., 1885, **9**, 577). They were carried out with solutions of globulin-free egg-albumin (about 1 grm. per 10 c.c.), to which were added 5 c.c. of 5% hydrochloric acid and a pepsin solution of definite strength; the solutions were then diluted to 100 c.c. and kept at 37·5° for 16 hours. The albumin was then removed from the solutions and the amounts of peptone formed determined by means of the optical rotations. In this way Schütz found the velocity of digestion to be proportional to the square-root of the concentration of the pepsin. The results of the first of the three series of experiments are given here:

Quantity of pepsin.	Rotation of the peptone in minutes.	
	Observed (mean).	Calculated.
1	7·3	7·4
2	9·75	10·4
3	12·8	12·7
4	14·8	14·7
5	16·5	16·4
6	18·45	18·9

Mention must also be made of the experiments of Borissow with trypsin and of Samojloff with pepsin, these experimenters also arriving at the relation $x=\kappa\sqrt{Et}$. (Dissertation, St. Petersburg, 1901; Arch. des Sci. Biol., 1893, **2**, 699; see Pawlow, Arbeit der Verdauungsdrüsen); there was here no intention of obtaining a representation of the chemical dynamics of enzyme action and, owing to the experimental methods employed in these investigations, no conclusions concerning this can be drawn. The same may be said of Walther's researches (Arch. des Sci. Biol., 1899, **7**, 15).

Very extensive numerical data on the digestion of protein by pepsin were given in 1895 by J. Sjöqvist (Skand. Arch. f. Physiol., 1895, **5**, 317), who followed the course of the digestion by measuring the electrical conductivity. Every 100 c.c. of solution, 0·05N with reference to hydrochloric acid, contained 2·23 grms. of albumin (almost freed from salts by dialysis) and also 2·5, 5, 10 or 20 c.c. of pepsin solution. The conductivity of these solutions fell during the experiment from the initial value $\mu=188\cdot4$ (old units) to a final value of about 83·4. The amount of albumin acted on, x, was taken as proportional to the fall Δ in the conductivity. The following tables contain the observed values of Δ at 37°, together with the corresponding

2·5 c.c. of pepsin solution per 100 c.c.

Hours.	Conductivity, μ	Change of conductivity, Δ	x	k_A	$k_s=\frac{x}{\sqrt{t}}$
0	188·4	—	—	—	—
0·5	—	—	—	—	—
1	—	—	—	—	—
2	177·3	11·1	10·5	2·97	7·45
4	171·1	17·3	16·41	3·78	8·21
6	167·4	21·0	19·93	3·81	8·13
8	164·5	(23·9)	22·68	3·77	8·02
9	163·1	25·3	24·00	3·82	7·90
12	159·9	28·5	27·04	3·70	7·70
16	156·4	(32·0)	30·36	3·62	7·59
20	152·9	35·5	33·68	3·70	7·53
32	146·2	42·2	40·04	3·40	7·08
48	139·8	(48·6)	45·06	3·20	6·50
64	135·0	(53·4)	50·78	3·13	6·34
96	127·9	60·5	57·41	2·80	5·87
∞				3·49	

proportional values of x, the calculated values of the constant κ_A of the Arrhenius formula (18) or (20) and, finally, the values of the constant of Schütz's formula $x = \kappa_S \sqrt{t}$.

5 c.c. of pepsin solution per 100 c.c.

Hours.	Conductivity, μ	Change of conductivity, Δ	x	κ_A	$\kappa_S = \frac{x}{\sqrt{t}}$
0	188.4	—	—	—	—
0·5	—	—	—	—	—
1	178·2	10·2	9·68	4·93	9·8
2	172·8	15·6	14·80	6·09	10·5
4	164·7	23·7	22·49	7·49	11·2
6	159·5	28·9	27·42	7·70	11·2
8	155·5	(32·9)	31·22	7·62	11·0
9	153·5	(34·9)	33·11	7·88	11·0
12	149·4	39·0	36·58	7·47	10·6
16	145·2	(43·2)	40·15	8·25	10·0
20	141·0	47·4	44·98	7·39	10·0
32	133·1	(55·3)	52·47	6·85	9·3
48	126·2	(62·2)	59·02	6·30	8·5
64	121·4	(67·0)	63·58	5·85	7·9
96	114·4	74·0	70·21	5·21	7·3
∞	—	—	—	6·84	

10 c.c. of pepsin solution per 100 c.c.

Hours.	Conductivity, μ	Change of conductivity, Δ	x	κ_A	$\kappa_S = \frac{x}{\sqrt{t}}$
0	188·4	—	—	—	—
0·5	179·2	9·2	8·73	8·04	12·35
1	174·2	14·2	13·47	10·34	13·47
2	165·9	22·5	21·35	13·40	15·10
4	154·8	33·6	31·88	16·30	15·94
6	148·0	40·4	38·34	16·65	16·01
8	143·2	(45·2)	42·88	16·42	15·31
9	140·8	47·6	45·14	16·51	15·05
12	136·1	52·3	49·62	14·09	14·32
16	130·9	(57·5)	54·56	15·23	13·64
20	125·7	62·7	59·50	15·44	13·30
32	119·4	69·0	65·46	12·90	11·60
48	113·1	75·3	71·45	11·25	10·31
64	109·1	(79·3)	75·25	10·08	9·41
96	101·8	86·6	82·17	9·60	8·39
∞	—	—	—	13·30	

20 c.c. of pepsin solution per 100 c.c.

Hours.	Conductivity, μ	Change of conductivity, Δ	x	κ_A	$\kappa_S = \frac{x}{\sqrt{t}}$
0	188·4	—	—	—	—
0·5	176·0	12·4	11·77	15·0	16·64
1	167·8	20·6	19·55	22·2	19·55
2	157·9	30·3	28·75	25·9	20·33
4	144·5	43·9	41·66	30·6	20·83
6	137·2	51·2	48·58	29·7	19·83
8	133·0	(55·4)	52·57	25·1	18·54
9	130·1	58·3	55·52	28·0	18·44
12	125·8	62·6	59·40	25·6	17·15
16	121·6	(66·8)	63·34	25·1	15·81
20	117·3	71·7	68·03	22·5	15·21
32	109·8	78·6	74·58	19·5	13·18
48	102·3	86·1	81·70	18·3	11·79
64	97·4	(91·0)	86·34	14·4	10·49
96	91·2	97·2	92·24	18·0	9·41
∞	—	—	—	22·7	

Sjöqvist's observations hence indicate that the relation $x = \kappa_S\sqrt{t}$ holds moderately well during the first half of the reaction.

Calculation of the constants for the various concentrations of enzyme E shows that these are approximately in the proportions, $\sqrt{0{\cdot}2} : \sqrt{0{\cdot}1} : \sqrt{0{\cdot}05} : \sqrt{0{\cdot}025}$.

κ_S	7·6	10·4	14·4	18·2
$\sqrt{E}$	0·500	0·707	1·00	1·42
Quotient $\kappa_S : \sqrt{E}$	15·2	14·7	14·4	12·8

So that, for small and equal values of t, Schütz's rule holds, i.e., the quantity of substance transformed is inversely proportional to the square-root of the concentration of the enzyme. The proportionality between the amount of albumin hydrolysed and the square-root of Et (enzyme-concentration × time) is much more general, as is shown by the following table in which Arrhenius (Immunochemistry, p. 67) has collected together the diminutions of conductivity given by Sjöqvist.

Et=	0·05	0·1	0·2	0·4	0·8	1·6	3·2	4·8	6·4	9·6
E=0·025	11·1	17·3	23·9	32·0	42·2	53·4	—	—	—	—
0·05	10·2	15·6	23·7	32·9	43·2	55·3	67·0	74·0	—	—
0·01	9·2	14·2	22·5	33·6	45·2	57·5	69·0	75·3	79·3	86·6
0·02	—	12·4	20·6	30·3	43·7	55·4	66·8	73·6	78·6	86·1
Mean....	10·2	14·9	22·7	32·2	43·8	55·4	67·6	74·3	79·0	86·4
Calculated	11	15·6	22	31·1	44	—	—	—	—	—

Further investigation of peptic digestion is due to Julius Schütz (H., 1900, **30**, 1), who coagulated the undigested protein remaining after 15 hours and determined the nitrogen in the filtered liquids by Kjeldahl's method. At a temperature of 38°, he obtained the following quantities of hydrolysed protein ($x_{obs.}$), the values calculated from Schütz's rule being given in the final column.

Quantity of pepsin.	$10^4.x_{obs.}$	$10^4.x_{calc.}$
1	212	213
4	471	426
9	652	639
16	799	852
25	935	1065
36	1031	1278

In the same year E. Schütz and Huppert (Pflüg. Arch., 1900, **80**, 470) gave further data concerning peptic digestion. The decomposition products—termed secondary albumoses—of the protein were determined polarimetrically. "The quantities of secondary albumoses formed are proportional to the square-roots of the times." Further, the quantities of digested protein, the sum-totals of the intermediate products and the amounts of secondary albumoses, are in the same ratios as the amounts of protein employed, namely, 1 : 2 : 3 : 4.

E. Schütz and Huppert also investigated the influence of hydrochloric acid. Under certain conditions, secondary albumoses are formed in proportion to the quantity of protein, to the square-root of the time, and to the concentrations of pepsin and acid. The conditions for this rule to hold are a moderately rapid reaction and a concentration of acid not exceeding 0·2%.

If we denote the amount of secondary albumoses by S, that of albumin by A, the time by t, the concentration of hydrochloric acid by s, and the quantity of pepsin by P, the velocity with which the secondary albumoses are formed is expressed by

$$S = \kappa'' A \sqrt{t.P.s}.$$

It must be stated that objections have been raised by Sjöqvist (loc. cit.) to the methods used in these experiments.

Gross (Berl. klin. Wochens., 1908, **45**, 643) expressed the view that Schütz's rule does not hold for peptic digestion, but that the amount of digestion is proportional directly to the quantity of enzyme and inversely to the time of digestion. His experiments were carried out as follows: Increasing amounts of pepsin were added to constant quantities of an acid solution of casein, observation being then made in each case of the time when the whole of the casein was digested, i.e., when no turbidity was produced by addition of saturated sodium acetate solution. The observations were made at intervals of 10–20 seconds, the temperature being 40°.

The following series of results may be quoted:

Casein solution, 50 c.c.		Casein solution, 50 c.c.	
Gastric juice, c.c.	Digestion complete in minutes.	Grübler pepsin, (0·1%) c.c.	Digestion complete in minutes.
1·0	52·7	1·0	64·0
2·0	25·0	2·0	31·7
4·0	12·2	4·0	16·7
8·0	6·25	—	—

In order to test Gross's results, Kurt Meyer (Berl. klin. Wochens., 1908, **45**, 1485) made a number of experiments by Fuld's edestin method.[1] The quantity of pepsin in any tube was four times, and that of protein twice, that in the preceding tube. Meyer collects his results in two tables, of which one is given here:

1% edestin solution in 0·03 HCl. c.c.	1% pepsin solution (Grübler) in 0·03 HCl.			
	0·0025 c.c.	0·01 c.c.	0·04 c.c.	0·16 c.c.
0·1	+	−	−	−
0·2		+	−	−
0·4			+	−
0·8				+
1·6				
3·2				

[1] The method was so modified that the amounts of digestion could be obtained in one series of experiments; thus, series with increasing quantities of edestin and similar series with increasing quantities of pepsin were carried out.

Incipient turbidity is indicated by +.

No exact idea can be formed of the magnitude of the experimental error is these investigations. K. Meyer himself, however, draws the conclusion that Schütz's rule is valid for peptic digestion.

0·05 c.c. gastric juice.

Quantity of casein, c.c.	Time of digestion in minutes.	Quantity of casein, c.c.	Time of digestion in minutes.
5	6·3	10	13·3
6	7·5	12	14·3
7	8·7	14	17·0
8	10·0	16	21·2

For the sake of completeness it may be mentioned that Spriggs (Journ. of Physiol., 1902, **35**, 465) followed the course of pepsin-action by measurements of the viscosity with Ostwald's viscosimeter. Against these experiments the objection may, however, be raised that the relation of viscosity to the degree of protein hydrolysis is not sufficiently known, so that no safe conclusions can be drawn from the results of these measurements.

Weis (Medd. fra Carlsberg Lab., 1903, **5**, 127) has made a very thorough investigation of the action of vegetable proteases. He found that peptic action proceeds relatively rapidly, whilst the tryptic decomposition of the albumoses is more gradual; the two actions can, to some extent, be separated. From the results obtained, which are extremely difficult to deal with in detail, it is to be concluded that, for the proteolysis of vegetable protein—at any rate within a certain region of concentration—Schütz's rule appears to hold. According to the results given on p. 176, etc., the exponent of the enzyme-concentration increases, with increasing dilution of the enzyme, from 0·5 (Schütz's rule) to 1.

In the separate series of experiments, the amounts of substance transformed are proportional to the square-roots of the times. From the table on p. 183 of the above paper we extract the following numbers, the values of $x:\sqrt{t}$ being given in addition.

Amount of change.			Amount of change.		
Hours. t	Mgrms. N. x	Ratio. $x : \sqrt{t}$	Hours. t	Mgrms. N. x	Ratio. $x : \sqrt{t}$
1	5·34	5·34	1	5·80	5·80
2	8·42	5·96	2	8·54	6·04
3	9·82	5·67	3	(12·00)	5·50
4	11·92	5·96	4	11·34	5·67
5	12·98	5·81	5	12·94	5·79
6	13·70	5·59	6	13·32	5·44
9	17·22	5·74	9	14·20	4·73

The author has also calculated the results of the experiments in which W e i s varied the concentration a of the substrate (protein). The third column gives the constants k for unimolecular reactions; the fourth, the product ka and the fifth, the constant κ_s of the formula $x\sqrt{a}=\kappa_s$.

After 5 Hours [1]

Protein concentration. a	Amount of N transformed as percentage of total N. x	$k=\frac{1}{t}\cdot\log\frac{a}{a-x}$	$k.a.10^6$	κ_s
1%	36·2	0·00065	65	36·2
2	25·9	0·00043	86	36·6
3	20·3	0·00033	100	35·2
4	16·0	0·00025	100	32·0
5	13·2	0·00020	102	29·5

After 2 Hours [1]

Protein concentration. a	Amount of N transformed as percentage of total N. x	$k=\frac{1}{t}\cdot\log\frac{a}{a-x}$	$k.a.10^6$	κ_s
1%	22·0	0·00090	90	22·0
2	17·0	0·00067	134	24·0
3	13·1	0·00051	153	22·7
4	9·1	0·00035	140	18·2
5	7·9	0·00030	150	17·7

[1] The numbers in the two tables were obtained in two separate series of experiments and are hence not comparable.

It will be seen that the amount of protein changed diminishes with increasing values of a. Arrhenius (Immunochemistry, p. 85) explains this as follows: When, for example, 10% of the protein is digested, the absolute amount of the products of the reaction is doubled if the initial concentration a is doubled. But the velocity of reaction is inversely proportional to the absolute quantity of the products and, therefore, also to the initial concentration. According to what was stated on p. 133, the amount of change, expressed as a percentage of the total amount of protein, is hence approximately inversely proportional to the square-root of a, as is shown by the fifth columns of the two tables given above.

The work of Weis on vegetable proteinases deals also with the trypsins, that is, with those proteolytic enzymes which act in alkaline solution.

From the results of experiments carried out virtually by Mett's method, H. M. Vernon (Journ. of Physiol., 1901, **26**, 421) has drawn the conclusion that the digestion of fibrin by trypsin follows Schütz's rule if the times of digestion are corrected for the destruction of the trypsin in the soda solution.

Amount of enzyme-extract E in c.c.	Time of digestion t in minutes.	Corrected time of digestion in minutes.	$t \times \sqrt{E}$.
2	11·8	11·14	16·2
1	17·7	16·25	16·3
0·5	26·8	23·62	16·7
0·25	36·1	30·57	15·3
0·125	80·4	57·32	20·3
0·0625	176·0	93·57	23·4

As has long been known qualitatively, the concentration of the acid present in peptic digestions exerts a marked influence on the time-course of the reaction. This influence is expressed quantitatively in Schütz's formula mentioned on p. 179, according to which—with certain definite conditions of the enzyme- and substrate-concentrations—the velocity of reaction is proportional to the square-root of the concentration of the hydrochloric acid.

The optimal concentration of the hydrogen-ions has been the subject of a recent and thorough investigation by Sörensen (Biochem. Z., 1909, **21**, 288). In this work the concentration of the H-ions was determined electrometrically, the progress of the reaction being measured by the amounts of protein preciptable after different times by stannous chloride or tannic acid. The results are given in the following table, the "exponents of the hydrogen-ions," p_H, being given in the first column; by this Sörensen understands the logarithm to base 10 of the reciprocal of the normality-factor of the solution as regards hydrogen-ions. The concentration of these ions is given also in the ordinary form (column 2).

It will be seen that the acidity-optimum increases with the duration of the peptic action.

The influence of the hydrochloric acid must be explained, as already mentioned, by the protein hydrochloride being more readily acted on than the free protein. Further the enzyme itself may be in the form of a salt, this pepsin hydrochloride showing increased activity; this possibility has recently been emphasised by J. Loeb (Biochem. Z., 1909, **19**, 534) but we have no definite indications on this question. As the author has pointed out (Ergeb. der Physiol., 1907, **6**), the "pepsin-hydrochloric acid"—the existence of which has been so often assumed—can mean nothing but a pepsin salt of hydrochloric acid.

The older investigations of Borissow on tryptic digestion have already been mentioned.

L. Pollak (Hofm. Beitr., 1904, **6**, 95) also employed Mett's method to examine a tryptic enzyme, glutinase, which he isolated, and which acts on gelatine. He arrived at the result that the action of this enzyme does not correspond exactly with Schütz's rule, which, however, it approaches far more closely than does the mixture of enzymes of an ordinary pancreas infusion or Grübler's trypsin.

V. Henri and Larguier des Bancels have investigated the action of trypsin on gelatine (C. R., 1902, **136**, 1581). By regarding, as Sjöqvist did, the change of conductivity as proportional to the progress of the reaction, they obtained confirmation of the formula

$$k = \frac{1}{t} \cdot \log \frac{a}{a-x}.$$

Concentration of the hydrogen-ions.		Increase (in mgrms.) of the nitrogen non-precipitable by stannous chloride after t hours' digestion.								Increase (in mgrms.) of the nitrogen non-precipitable by tannin after t hours' digestion.					
p_H	Concentration.	$t=\frac{1}{2}$	1	2	4	8	13	20	49	$t=1{\cdot}5$	3	6	12	24	48
0·76	$1{\cdot}7.10^{-1}$	6·04	8·50	13·24	19·34	22·80	25·60	—	—	3·82	6·02	9·25	12·38	15·16	—
0·99	$1{\cdot}0.10^{-1}$	6·62	9·32	14·34	21·02	24·10	25·72	28·42	30·28	4·22	6·60	9·58	13·22	15·46	18·26
1·22	$6\ .10^{-2}$	9·04	12·26	17·74	—	27·50	27·74	28·80	31·32	5·08	7·46	10·46	13·92	16·22	18·94
1·63	$2\ .10^{-2}$	11·00	15·24	18·70	24·76	27·86	28·45	28·94	30·92	5·06	7·32	10·22	13·38	15·86	18·66
2·26	$5\ .10^{-3}$	10·82	15·00	17·50	22·06	25·14	26·42	27·08	28·08	4·22	6·20	8·86	11·64	14·62	17·32
4·09	$8\ .10^{-5}$	7·84	10·80	12·42	13·30	—	13·58	—	16·12	0·94	1·48	2·52	3·86	5·62	8·04

Since, however, the final value of the process is not observed (or, at any rate, not given) and the observations are only spread over the very short period of about an hour, the real decomposition of protein cannot have proceeded very far and these experiments tell little about the course of tryptic digestion. As has been calculated by Arrhenius, the conductivity is approximately proportional to the square-root of the time of digestion.

t (minutes)	10	20	30	40	50
Conductivity (mean)	27·3	44·0	53·0	58·7	65·7
$7{\cdot}37\sqrt{t}$	29·3	41·5	50·0	58·7	68·8

Of wider scope are the experiments of Bayliss (Arch. Sci. Biol. St. Petersburg, 1904, **11**, Supplement), in which also the conductivity method was employed. The substrate used was partly caseinate in faintly ammoniacal solution and partly gelatine. The experimental results are given mostly in the graphic form. It is found that the constants for unimolecular reactions diminish considerably with lapse of time, this being attributed to the retarding effect of the products of the reaction. The numbers obtained by Bayliss are indeed in good agreement with the formula,

$$a\ln\frac{a}{a-x}-x=\kappa Et.$$

With concentrations of casein up to about 4%, the velocity of digestion is proportional to the concentration of the substrate; in 4–8% solutions, the velocity is independent of the concentration of the substrate, whilst with more than 8%, inverse proportionality sets in. As regards the relation between the velocity and the concentration of the enzyme, approximate proportionality exists during the first quarter of the reaction; but even in the second quarter, the velocity of reaction is considerably less than would correspond with the concentration of the trypsin (loc. cit., p. 26).

Using Volhard's method for estimating pepsin and trypsin—which will be referred to in the Appendix— W. Löhlein (Hofm. Beitr., 1905, **7**, 120) arrived at the result that tryptic digestion does not follow Schütz's rule. O. Faubel's results (Hofm. Beitr., 1907, **10**, 35) point to the same conclusion.

The measurements which have as yet been made do not indicate, with the certainty that might be desired, the con-

ditions for simple proportionality between velocity of reaction and the concentration of the enzyme. But far more often than in experiments with pepsin have the conditions been such that the relations:

$$\text{Amount of digested protein} = \text{const.}\sqrt{E},$$

and

$$\text{Amount of digested protein} = \text{const.}\sqrt{t},$$

have been found to hold only over a very limited range.

But, as with pepsin (cf. p. 179), it appears to be quite general that the same quantity a of protein is digested if the amonut of enzyme is made to vary inversely with the time, i.e.

$$x = \text{const. } f(E.t). \quad . \quad . \quad . \quad . \quad . \quad . \quad (22)$$

This rule evidently holds only for those cases in which the proportionality between x and Et is direct. This occurs with undisturbed unimolecular catalytic reactions, so long as the products of the reaction x are small in amount compared with the substrate a. Further, according to the measurements of Bayliss, Hedin and others, this rule is obeyed, in the case of trypsin, with small quantities of enzyme and also with conditions so chosen that

$$x = \text{const. } \sqrt{Et}.$$

This rule is contained in the widely-used formula (18), as is shown by the derivation of the latter.

Formula (22) evidently means that the progress of the digestion depends only on the quantity of protein already hydrolysed, no matter whether this has been obtained by the action of much enzyme for a short time or by that of less enzyme for a longer time.

This relation has been confirmed by Hedin (Journ. of Physiol., 1905, **32**, 468; ibid, 1906, **34**, 370; H., 1908, **57**, 471). One of the tables from the last of these papers is given below.

The amount of casein digested was measured by the quantity of nitrogen not precipitated by tannic acid. The values given in the various columns under $E.t$ indicate the numbers of tenths of a c.c. of normal acid required to neutralise the ammonia obtained by Kjel-

dahl's method from equal volumes of the filtrate from the tannin precipitate:

Et.....	1·0	2·0	2·5	5·0	7·5	10·0	15·0	20·0
1.....	5·8	10·35	12·95	20·15	24·50	[26·95	31·0	34·05
2.....	5·85	10·75	13·25	20·40	24·65	27·8	31·7	—
3.....	5·70	10 85	13·35	20·30	24·35	27·0	31·05	33·75
4.....	6·10	10·75	13·15	19·90	23·80	26·95	30·75	33·55

As regards the investigation of the kinetics of the digestive enzymes, the difficulties which beset all enzymic problems are here supplemented by others due to the complicated nature of the proteins. Observations made by any of the physico-chemical methods, whether optical or electrical, represent the effect of a large number of decompositions proceeding simultaneously, and it is, as the author has repeatedly emphasised, surprising that simple direct relations are so often found. The processes of decomposition would hence become decidedly more apparent if the dipeptides, which Fischer's work has rendered accessible, were employed as substrates. The first experiments in this direction were made by Euler (H., 1907, **51**, 213) on glycylglycine, not however with the trypsin of the pancreas, but with the proteolytic enzyme of the walls of the intestines, the so-called erepsin.

The course of the reaction was followed by the change in conductivity of an alkaline solution of glycylglycine. The final conductivity, obtainable by complete hydrolysis of the glycylglycine, was determined beforehand with glycine solutions of corresponding concentration. In the possibility of a certain knowledge of this final value seems to lie one of the principal advantages of using dipeptides as substrates in tryptic and ereptic decompositions. It was also found that the diminution of the conductivity is proportional to the diminution of the concentration of the dipeptide.[1]

The first result obtained was that the velocity of hydrolysis of glycylglycine depends greatly on the alkalinity of the erepsin solution.[2]

[1] It is to be noted that the existence of such proportionality, which forms the basis of further calculations, was not shown in previous investigations where tryptic or peptic digestion of protein was followed by means of the conductivity.

[2] The alkalinity is expressed as the normality of the total soda added. The actual alkalinity, that is, the concentration of the free base, is very much smaller, since the greater part of the soda is used up in neutralising the glycylglycine.

N/10-glycylglycine. 5 grms. powdered erepsin per 100 c.c.

Concentration of alkali	0	0·04	0·05	0·075	0·10
Reaction constant×1000	<0·05	7·0	6·2	2·6	0·2

Different preparations of the enzyme showed varying sensitiveness towards alkali.

It was next ascertained that the decomposition of glycylglycine is a reaction of the first order and that, under favourable conditions, the corresponding velocity coefficients k remain constant until the reaction is half completed.[1]

N/10-glycylglycine. 5 grms. powdered erepsin per 100 c.c.

a. 0·04 N-NaOH.			*b.* 0·05 N-NaOH.		
Minutes.	$1000(a-x)$.	$1000k$.	Minutes.	$1000(a-x)$.	$1000k$.
0	920	—	0	935	—
7	819	7·20	10	806	6·46
15	721	7·08	17	739	6·00
22	649	6·88	25	654	6·18
30	579	6·70	30	622	5·90

In most cases a considerable fall in the velocity occurs even after about half an hour. This depends principally on destruction of the erepsin, the rapidity of the destruction increasing with the amount of free alkali in the solution.

Within certain limits the velocity of reaction is almost independent of the concentration of the dipeptide. This independency holds, however, only for certain relations between the concentrations of the enzyme and substrate. With small amounts of enzyme, the velocity of reaction rises with increase of the concentration of the glycylglycine.

0·1 N-glycylglycine; 0·04 N-NaOH. $1000k = 0·35$ 0·2 N-glycylglycine; 0·05 N-NaOH. $1000k = 0·55$

Since, as has already been mentioned, the concentration of free alkali has here also a considerable influence on the velocity of reaction, the influence of the concentration of the substrate is very difficult to explain. The author concludes, from the results

[1] As the behaviour of erepsin, like that of other enzymes, depends on the absolute activity of the solution, it is advisable, in order to make differene results comparable, to establish a standard action. A normal preparation might be taken as one which decomposes glycylglycine or an optically active dipeptide, in 5% solution, to the extent of one-half in 1 hour.

of these experiments, that only the alkali salt of the dipeptide undergoes hydrolysis (Arkiv för Kemi, 1907, **2**, No. 39).

As regards the effect of the concentration of enzyme, it may be said that, in most of the conditions of concentration examined, the velocities of reaction were proportional to the enzyme-concentrations; the Schütz-Borissow law did not hold in any instance. The following numbers serve as examples:

Concentration of erepsin	5	4	3	2
$1000k$:	6·5	5·4	4·3	2·8

With low concentrations of the enzyme, especially with feeble preparations of pancreatin, k increases far more rapidly than the concentration of the enzyme.

With relatively high concentrations of enzyme, deviations from proportionality in the sense of Schütz's rule do, indeed, occur; but the experimental errors are so great in these reactions, which take place in a few minutes, that conclusions cannot be drawn from the results.

If optically active polypeptides are employed as substrates, the enzymic hydrolysis can often be followed polarimetrically. Such measurements have been carried out by Abderhalden and his collaborators.

The following results, obtained with *d*-alanyl-*d*-alanine, indicate the time-law of this reaction (Abderhalden and Koelker, H., 1907, **51**, 294; Abderhalden and Michaelis, H., 1907, **52**, 326):

0·45 grm. dipeptide +6 c.c. pressed juice.					0·45 grm. dipeptide +4 c.c. pressed juice +2 c.c. physiological salt solution.				
Min.	Rotation	x	$\frac{10^2x}{t}$	$k.10^4$.	Min.	Rotation.	x	$\frac{10^2x}{t}$	$k.10^4$.
0	−1·21°	—	—	—	0	−1·31°	—	—	—
3	−0·96	0·39	130	453	3	−1·17	0·18	60	192
7	−0·74	0·59	84	390	7	−0·98	0·37	53	183
11	−0·51	0·84	71	382	10	−0·81	0·54	54	202
18	−0·20	1·15	64	380	16	−0·56	0·79	49	214
20	−0·16	1·19	59	373	25	−0·21	1·14	46	268
27	+0·01	1·36	50	451	30	−0·09	1·26	42	294
34	+0·05	1·40	41	430	35	+0·02	1·37	—	359
40	+0·07	1·42	35	—	43	+0·07	1·42	—	—
55	+0·10	1·45	—	—	54	+0·08	1·43	—	—
65	+0·10	1·45	—	—					

0·45 grm. dipeptide+3 c.c. pressed juice+3 c.c. physiological salt solution.

Minutes.	Rotation.	x	$\frac{10^3 x}{t}$	$k.10^4$
0·0	−1·31°	—	—	—
5·0	−1·16	0·19	380	125
6·5	−1·09	0·26	400	132
7·5	−1·05	0·30	400	134
16·0	−0·76	0·59	369	142
22·0	−0·54	0·81	368	161
28·0	−0·32	1·03	368	192
30·0	−0·25	1·10	314	209
38·0	−0·09	1·28	332	232
45·0	+0·01	1·36	—	265
60·0	+0·07	1·42	—	—

0·6 grm. *d*-Alanyl-*d*-alanine in 7·6 c.c. pressed juice +0·4 c.c. physiological salt solution.

Min.	Rotation	x	$\frac{x}{t}$	$k.10^4$
0	−1·30°	0	—	—
5	−1·08	22	44	148
12	−0·85	45	38	140
19	−0·59	71	38	162
26	−0·23	107	40	241
30	−0·09	121	40	289
			40	

0·6 grm. *d*-Alanyl-*d*-alanine +5·7 c.c. pressed juice +3·3 c.c. physiological salt solution.

Min.	Rotation.	x	$\frac{x}{t}$	$k.10^4$
0	−1·23°	0	—	—
7	−1·09	21	30	60
13	−0·91	39	30	92
26	−0·46	84	32	144
37	−0·11	119	32	216
50	—	140	28	—
			30	

It will be seen that the theoretical formula, $k = \frac{1}{t} \cdot \log \frac{a}{a-x}$, leads to reaction-coefficients which show a continuous rise, the amount of this increasing with diminution of the amount of enzyme for a constant amount of substrate.

On the other hand, with relatively small amounts of enzyme, the value of the expression $\frac{x}{t}$ is strikingly constant, and this appears to indicate that the change proceeds independently of the amount of substrate still to be decomposed. But it is more probable that here, as is always the case, the velocity of reaction, $dx : dt$, is proportional to the quantity of substrate present, and that the reaction, as it proceeds, is subjected to some secondary acceleration.

The velocity of reaction is approximately proportional to the amount of enzyme:

$$40 : 7{\cdot}6 = 5{\cdot}4,$$
$$30 : 5{\cdot}7 = 5{\cdot}4.$$

This proportionality is much less sharp with *d*-alanylglycine, with which A b d e r h a l d e n and K o e l k e r (H., 1908, **55**, 416) have experimented; the two following tables appear on p. 422 of this paper:

2 c.c. Dipeptide solution = 0·001 normal. 0·5 c c. pressed yeast juice. 4 c.c. water.			2 c.c. Dipeptide solution = 0·001 normal. 2·0 c.c. pressed yeast juice. 2·5 c.c. water.		
Minutes.	Rotation.	$k.10^4$.	Minutes.	Rotation.	$k.10^4$.
0	+0·85° (extrap.)	—	0	+0·85° (extrap.)	—
12	0·80	22	12	0·70	70
31	0·71	25	31	0·54	64
62	0·63	22	62	0·38	56
86	0·53	24	86	0·28	56
118	0·49	23	118	0·19	55
190	0·36	20	—	—	—

Here, therefore, the velocity of reaction increases considerably more slowly than the concentration of the enzyme, but there is no indication of the validity of S c h ü t z 's law.

With reference to the effect of the concentration of the substrate, A b d e r h a l d e n and K o e l k e r (H., 1908, **54**, 363) have made experiments with active material, the following table giving some of their results:

Minutes.	($\frac{3}{320}$-mol.) 3·0 c.c. *d*-Alanyl-*d*-alanine solution, 1·0 c.c. pressed yeast juice. 2·0 c.c. water. Angle.	($\frac{3}{240}$-mol.) 4·0 c.c. *d*-Alanyl-*d*-alanine solution, 1·0 c.c. pressed yeast juice. 1·0 c.c. water. Angle.	($\frac{3}{200}$-mol.) 5·0 c.c. *d*-Alanyl-*d*-alanine solution, 1·0 c.c. pressed yeast juice. Angle.
4	−1·36°	−1·83°	−2·15°
12	−1·31	−1·75	−2·10
32	−1·17	−1·58	−1·97
61	−0·95	−1·31	−1·73
92	−0·67	−0·97	−1·43
128	−0·42	−0·69	−1·16
167	−0·18	−0·38	−0·86
190	−0·08	−0·23	−0·69
238	+0·03	+0·01	−0·35
308	+0·05	+0·13	+0·02
357	+0·05	+0·12	+0·15
377	+0·11	+0·18	+0·29

The dipeptide employed in these experiments was, as these investigators state, not free from the racemic compound, so that the course of the reaction and the calculations become more complicated. It can, however, be seen from the above numbers that the amounts of dipeptide hydrolysed in a certain time are, as a first approximation, independent of the initial concentration of the dipeptide. The velocity constants calculated according to the unimolecular formula would, therefore, diminish considerably with increasing concentration of the dipeptide instead of being independent of this concentration, as theoretically they should be. Within a certain region of concentration or with a certain ratio between the concentrations of substrate and enzyme, this relation is found in the case of most enzymes. It has already been shown, by experiments made by the a u t h o r , how greatly the degree and character of the reaction change with the alkalinity of the solution, and confirmation of this result is afforded by the investigations of A b d e r h a l d e n and K o e l k e r (loc. cit., p. 378). To obtain definite results in any study of the kinetics of trypsin and erepsin, careful attention must be paid to the alkali-content of the solutions. If, as the above measurements indicate, it is the a l k a l i s a l t s of the dipeptides or proteins which undergo hydrolysis and thus function as the "active molecules," the concentration of the substrate is not merely that of the dipeptide or protein but depends also on that of the alkali added; hence no general simple relations for the velocity of reaction will be found if the concentration of the dipeptide or protein alone is varied. It is of more value to ascertain how the concentration of the alkali must be altered at the same time for the theoretical requirement—independence of the reaction constant on the concentration of the substrate—to be fulfilled.

A b d e r h a l d e n and K o e l k e r (H., 1908, **55**, 416) have examined also the course of the reaction in the case of tri- and tetra-peptides. Study of such higher polypeptides not only serves to characterise the polypeptides, with which the velocity of hydrolysis is a characteristic constant, but they are also of interest in indicating the course of the reaction when, as happens with the true proteins, several hydrolyses take place at the same time. As examples are given the results of two series of experiments on *d*-alanylglycylglycine; since the separate observations

are subject to considerable errors, the results given here have been interpolated graphically.

3·32 c.c. Peptide solution = 0·002 mol. 1·0 c.c. pressed yeast juice. 2·18 c.c. H_2O.			4·98 c.c. Peptide solution = 0·003 mol. 1·0 c.c. pressed yeast juice. 0·52 c.c. H_2O.		
Minutes.	Angle (interp.)	$k.10^4$.	Minutes.	Angle (interp.)	$k.10^4$.
0	1·70°	—	0	2·55°	—
20	1·15	85	20	1·80	76
40	0·70	96	40	1·20	82
60	0·41	103	60	0·80	83
80	0·20	116	80	0·55	83
			120	0·26	82

It will be seen that the constants calculated from the unimolecular formula and given in the third column, do not increase very greatly with the time.

A dynamic investigation of the vegetable ereptases discovered by Vines (Annals of Bot., 1910, **24**, 213), would be of considerable interest.

At about the same time as the author, A. E. Taylor carried out experiments on alkaline proteolysis, using a chemically definite substrate in order to render the results more definite. To this end he prepared protamine sulphate from the salmon by Kossel's method and hydrolysed it with Grübler's pancreatin. Unfortunately the results of these researches are given only very briefly (Berkeley: On Fermentation, 1907, p. 152). The numbers do, however, show that the law $x = \kappa\sqrt{t}$ does not hold for trypsin. The results of each series of experiments can be calculated by the formula for unimolecular reactions: but the constants vary if at different times the concentration of the substrate is altered. Further, under constant external conditions, the velocity of protamine digestion is simply and directly proportional to the concentration of the trypsin. These results were obtained with the optimal concentration of hydroxyl-ions.

Protamine, 0·100 grm. in 50 c.c. Temperature 40°

Quantity of trypsin, grm.	0·008	0·006	0·004	0·003	0·002	0·001	0·0005
Mean time of digestion, t	37	50	76·5	103	156	313	657
Quantity of trypsin $\times t$	296	300	306	309	312	313	328

Here also, then, the relation $x = \kappa_3\sqrt{E}$ does not hold.

In connection with the above investigations on the action of proteolytic enzymes in vitro, reference may be made to the relations deduced by Arrhenius (H., 1909, **63**, 323) from the results of the numerous experiments made by E. S. London on digestion in the stomach of the dog.

For the experimental details reference must be made to London's original papers (H., 1905, **45**, 381; **46**, 209; 1906, **47**, 368; **49**, 324; **50**, 125; 1907, **51**, 241, 468; **52**, 482; **53**, 148, 240, 246, 326, 334, 356, 403, 429; **54**, 80; 1908, **55**, 447; **56**, 378–416, 512–553; **57**, 113, 529; 1909, **60**, 191–283; **61**, 69; **62**, 443–464; 1910, **65**, 189–218; **68**, 346–380). The following cases are to be distinguished:

1. Digestion of different quantities of meat introduced by the mouth. The fact, established by London, that the amount digested in a given time is not proportional to the quantity of nutriment taken, is explained as follows:

"The first 100 grms. of meat lie close to the stomach-wall in a layer of uniform thickness. Within this lies another layer of 100 grms. of meat, this being rather thicker, as its surface diminishes approximately as the cross-section of a cone as the apex is approached. Further layers of 100 grms. follow, the thickness continually increasing. Into these layers the gastric juice diffuses from the mucous membrane. The quantity of gastric juice in each layer diminishes considerably and the diminution increases in extent as the layers lie farther away from the mucous membrane. This is conditioned partly by their increasing thickness and partly by the laws of diffusion, which require such increasing diminution even for layers of equal thickness. Hence it comes about that the innermost layers are not perceptibly digested and the amount digested in a given time increases, as the amount of food taken is augmented, only to a maximum."

As far as digestion is concerned, every layer is independent on every other and the total quantity digested is the sum of those digested in the various layers. On the simple assumption that the digestion in the outermost layer is always the same, the number of layers which may lie inside has been calculated by Arrhenius from London's results by means of an

empirical formula. The latter gives the quantities of food digested in a certain time in the various layers and corresponds very well with the experimental figures.

With the aid of this formula Arrhenius has prepared a table showing the time-course of the digestion which he represents also graphically (Fig. 5).[1]

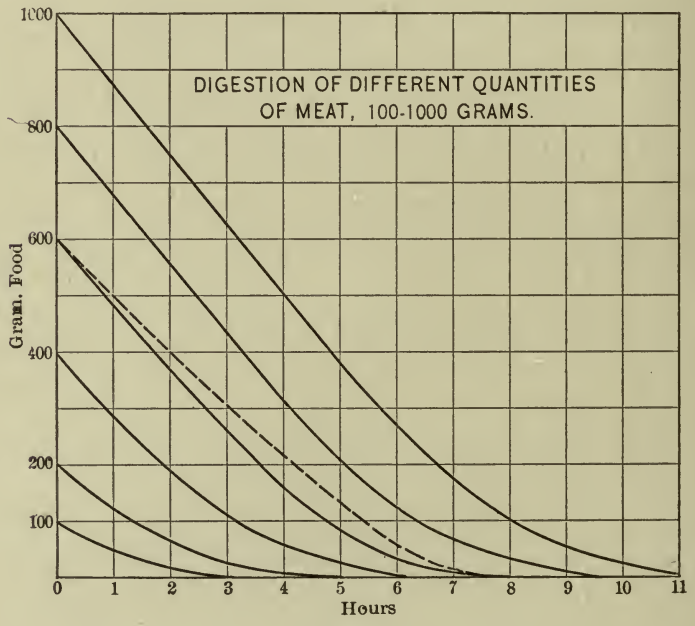


FIG. 5.

[1] From Chigin and Lobassow's results (Dissertation, St. Petersburg, 1896), Herzog (Zeitschr. f. allg. Physiol., 1904, **4**, 163) has attempted to calculate the time-course of the secretion of gastric juice.

Determinations were made of the quantities of pepsin (measured by the number of c.c. of gastric juice and its specific digestive power) secreted in a blind sac in the fundus part of the stomach, when food was introduced into the stomach. According to Herzog's calculations, the secretion of pepsin follows the formula for unimolecular reactions.

With introduction of solid food the values of k obtained are moderately constant. But in what degree the measurement and calculation of the quantities of pepsin by the Russian workers are free from objection, cannot well be judged. Chigin and Lobassow's results have recently been referred to in a paper by Arrhenius (H., 1909, 63, 323).

From the curve for 1000 grms. the quantities remaining undigested after a certain number of hours have been calculated, the results being as follows:

Time in hours:	0	1	2	3	4	5	6	7	8	9	10	11	11·5
Amount undigested:	1000	875	750	627	507	390	276	180	100	51	24	7	3
Difference per hour:		125	125	123	120	117	114	96	80	49	27	17	

It will therefore be seen that the quantity digested is at first very nearly proportional to the time, but gradually diminishes later.

This apparent contradiction of Schütz's rule depends on the fact that in vivo the products of digestion are continually removed, whilst in vitro they remain in the solution and hence retard the reaction.

The above table leads to a very important rule. It will be seen that for practically complete digestion of 1000 grms. of meat 11·5 hours are necessary. The time t required to digest another quantity M is calculated by subtracting from 11·5 hours the time taken to digest (1000–M) grms. of meat when the initial quantity is 1000 grms. In this way the times $t_{obs.}$ given below for varying values of M have been obtained.

M	$t_{obs.}$	$t_{calc.}$	$(t_{obs.} - t_{calc.})$
1000	11·50	10·81	+0·69
900	10·70	10·26	+0·44
800	9·90	9·67	+0·23
700	9·09	9·05	+0·04
600	8·27	8·38	−0·11
500	7·44	7·65	−0·21
400	6·60	6·84	−0·24
300	5·73	5·92	−0·19
200	4·74	4·84	−0·10
100	3·45	3·42	+0·03
50	2·40	2·42	−0·02
25	1·55	1·71	−0·16

Besides the values of $t_{obs.}$, those of $t_{calc.}$ are also given, these being calculated from the formula

$$t = 0{\cdot}342\sqrt{M}.$$

Hence, the time required for the virtually complete digestion of flesh-food introduced per os is very nearly proportional to the square-root of the quantity of food.

This square-root law is repeated in numerous series of experiments and also with different nutriment. It holds not only for ordinary meat but, as was shown by Arrhenius, for the digestion of gliadin (London, H., 1908, **56**, 394), of the albumin of hens' eggs (London, H., 1908, **56**, 405) and of bread (London, H., 1906, **49**, 359), and is therefore of the greatest importance for calculating the digestion periods of solid foods.

2. Digestion of meat introduced directly into the stomach by a fistula. If the nutriment is taken per os, a reflex secretion of gastric juice occurs, so that the digestion is greatly accelerated. When, however, the introduction of food takes place through a fistula, the secretion of gastric juice produced by the stimulating action of the meat on the stomach-wall is at first gradual and increases with the time; indeed, as the following calculation shows, the quantity of gastric juice in the stomach is about proportional to the time elapsing since the introduction of the meat.

Thus, if the digestion is proportional to the quantity of gastric juice present and the latter to the time, the amount digested per unit of time, $\frac{dx}{dt}$, is proportional to the time t and to the quantity of meat $(M-x)$ still present (M being the amount initially added), or, mathematically:

$$\frac{dx}{dt} = 2k.t(M-x),$$

from which follows:

$$\log \frac{M}{M-x} = kt^2.$$

London and Pewsner's results, given below, have been calculated according to this formula.

A. Feeding of dog per fistulam. Eyes and nose covered.

t (hours)	Undigested quantity $(M-x)$	$(M-x)_{calc.}$	Difference.
0	100	100	—
2	84	84·7	−0·7
4	56	51·5	+4·5
6	20	22·5	−2·5
8	7	7·0	0
9	0	3·5	−3·5

B. Feeding per fistulam. Eyes and nose uncovered.

t (hours).	Undigested quantity $(M-x)$.	$(M-x)_{calc.}$	Difference.
0	100	100	—
2	84	83·7	+0·3
4	53	49·1	+3·9
6	18	20·1	−2·1
8	5	5·8	−0·8
9	0	2·7	−2·7

In the experiment *A*, the constant had the value $k=0·0180$ and in *B*, $k=0·0193$.

3. If we now ask how the total course of the digestion is to be represented (i.e., not only as regards the time necessary for local digestion), it may in general be stated that, in so far as small quantities of food (the layer which, according to the above assumption, is in immediate contact with the stomach-wall) are concerned, digestion follows the law holding for unimolecular reactions. This is shown by the following examples:

Digestion of boiled protein, 200 grms.				Digestion of meat, 200 grms.			
t	$(100-x)$	$(100-x)_{calc.}$	Diff.	t	$(100-x)$	$(100-x)_{calc.}$	Diff.
0	100	100	—	0	100	100	—
1	70	65	+5	1	60	56	+4
2	32	42	−10	2	31	32	−1
3	28	27	+1	3	15	18	−3
4	18	18	0	4	5	10	−5
5	15	12	+3	5	0	6	−6
6	4	7	−3				
(7	0	5	−5)				

By $(a-x)$ is denoted the quantity of undigested protein still in the stomach at the time t. The calculated figures are obtained by means of the formula:

$$k=\frac{1}{t}\cdot\log\frac{100}{100-x}.$$

The same formula holds for the digestion of dissolved carbohydrates (amylodextrin).

4. The resorption of a sugar solution in the intestines also follows the unimolecular law.

If a solution of glucose is introduced into the intestine, the latter gives up water to it if the solution is concentrated, but takes up water from a dilute solution. The quantity of water yielded to the solution reaches a maximum, amounting to 900 c.c., corresponding with the capacity of the body to give up water From London's results, Arrhenius has calculated the concentration of the solution when no water is taken up o· give out by the intestine, this being 10·5% of glucose. As was to be expected, it is found that the amount of water taken from the intestine is proportional to the excess of the concentration over 10·5%. The law followed is hence expressed by the equation:

$$\log\frac{900}{900-W}=k(C-10\cdot5)t,$$

where W is the amount of water given up by the intestine and $(C-10\cdot5)$ represents the excess of the concentration over 10·5%.

RENNET (CHYMOSIN)

By the action of rennet, casein is converted into paracasein (compare p. 45); whether at the same time some component, possibly an albumose, is split from the casein molecule is a disputed question. On account of this supposed partial hydrolysis, and of the occurrence of pepsin and chymosin together, the latter is classed with the true proteolytic enzymes.

It must here be borne in mind that the clotting of milk by rennet consists of two processes (Hammarsten): chymosin only accelerates the conversion of casein into paracasein, a change which proceeds without calcium salts. These salts are necessary only for the precipitation of the curd (paracasein) for which, indeed, no rennet is required.

It would therefore be expected that the time which clotting takes to begin is made up of two periods: (1), that necessary for

the casein to be transformed, almost completely, into paracasein—time of conversion, and (2), that necessary for the formation of a visible clot—time of separation. Such an assumption was advanced by Fuld (Hofm. Beitr., 1902, **2**, 169). According to this author, the time of separation amounts to several days or to a few minutes or even less, in dependence on the temperature of the paracasein solution, and is so inappreciable in comparison with the long period of conversion of the experiments referred to above, that it may be unhesitatingly neglected. On the other hand, Reichel and Spiro, in their most recent investigation, regard such an assumption as that of Fuld as unjustified, and express the view that, with reference to its time-course, the whole clotting process must be considered as a single process (Hofm. Beitr., 1906, **8**, 15).

According to Bang (Skand. Arch. f. Physiol., 1911, **25**, 105), the clotting of milk by rennet is an extremely complicated process, in which the calcium salts present are distributed between organic acids, lactalbumin and lactoglobulin, whilst, on the other hand, the casein is distributed among the basic constituents of the solution.

The clotting process obeys the law, $[E].t = \text{const.}$, or

The product of the enzyme-concentration $[E]$ and time of clotting t is constant.

This relation was first observed by Segelcke and Storch (Ugeskrift för Landmaend, 1870) and was subsequently fully confirmed by Hansen and Soxhlet (Milchzeitung, 1877). The most complete investigations of the action of rennet are due to Fuld and to Reichel and Spiro.

At 40°, E. Fuld (Hofm. Beitr., 1902, **2**, 172) obtained the following numbers, which proved the exact and general validity of the time-law even with small amounts of rennet:

Quantity of rennet.	Time of clotting, t, in seconds.	Product.
0·4	6	24
0·4	6·6	26
0·2	13	26
0·1	25	25
0·8	6	48
0·4	17	44
0·2	22	44
0·1	45	45

According to C. Gerber (Soc. Biol., 1907, **63**, 575), who has recently made a thorough study of the clotting process, it is essential, when working with the rennet of commercial pepsin, to employ temperatures between 25 and 30° so that the enzyme shall be under normal conditions. Within these limits of temperature, Gerber finds that the law of Segelcke and Storch holds closely for parachymosin.

Drops of parachymosin E....	1	2	3	4	6	7	8	9	10
Time of clotting, t.....	29·66′	14·75′	10·30′	7·60′	5·50′	4·50′	3·66′	3·16′	2·92′
$E.\ t$.....	29·6	29·5	31	30·3	33	31·5	29·3	28·4	29·2

Madsen has also investigated the duration of milk-clotting, the method used being similar to that employed with pepsin action (see Arrhenius, Immunochemistry, p. 72).

He adds, for instance, varying quantities of rennet to equal amounts of milk in test-tubes, places the mixtures in a water-bath at a definite temperature, takes them out after time t, cools them quickly and determines the smallest quantity of rennet L which has produced clotting.

Coagulation of milk by rennet solutions of different concentrations at 36·55°

Minutes.	L.	Lt.	Minutes.	L.	Lt.
4	0·08	0·32	35	0·007	0·25
6	0·05	0·30	50	0·005	0·25
9	0·033	0·30	70	0·004	0·28
11	0·024	0·26	80	0·0032	0·26
12	0·019	0·23	100	0·0028	0·28
14	0·0175	0·25	120	0·0025	0·30
20	0·013	0·26	180	0·00185	0·33
25	0·010	0·25	240	0·0017	0·41
30	0·007	0·21			

Bang (Pflüg. Arch., 1900, **79**, 425) found that the relation, $[E]\ t = \text{const.}$, did not hold for parachymosin.

By a series of careful experiments Reichel and Spiro (Hofm. Beitr., 1905, **7**, 485) showed that the relation between quantity of rennet L and period of clotting t can be expressed generally by the formula:

$$L^n.t = \text{const.}$$

The exponent n of L changes with the nature and amount of the other substances in the solution, and in a liquid of the composition of milk it assumes exactly the value 1, so that the law holds in its simplest form,

$$L.t = \text{const.}$$

In the following experiments the solutions were prepared by dilution with whey, the clotting action of which was negligible in comparison with that of the enzyme added.

Milk.	c.c. of 35% rennet diluted with whey.	Whey.	Time of clotting t in seconds.		
			Whey I.	Whey II.	Whey III.
0·5	0·5	4·0	22	22	33
1·0	0·5	3·5	17	22·5	27
1·5	0·5	3·0	18	27	28
2·0	0·5	2·5	17	25·5	26·5
2·5	0·5	2·0	18	24·5	25·5
3·0	0·5	1·5	16	26	26
3·5	0·5	1·0	16	26	24
4·0	0·5	0·5	15	25	26
4·5	0·5	—	16	24	27

As will be seen from these results, the constant Lt or, since L is the same in all cases, the time of clotting, is independent of the concentration of milk (casein) in the solution from 20% up to 90 %.

Replacement of the whey by 0·9% sodium chloride solution resulted in constancy of the time of clotting for more dilute as well as for more concentrated solutions.

More extended investigations then showed that the difference between the times of clotting for dilute and concentrated milks was approximately proportional to the difference between the dilutions. Hence, if V denotes the volume of the diluted milk, M that of the undiluted milk, and t and t' the times of clotting in the two media, then:

$$(t-t')\left(\frac{M}{V-M}\right) = \text{const.}$$

This is shown by an extract from Table VII of Reichel and Spiro's paper.

Rennet.	Milk.	Whey.	Time.	$(t-t')\left(\frac{M}{V-M}\right)$
1·0	0·2	8·8	110	1·60
1·0	0·6	8·4	50	1·81
1·0	1·0	8·0	39	1·93
1·0	2·0	7·0	28·6	1·75
1·0	4·0	5·0	24	1·60
1·0	8·0	1·0	22	1·60

The influence of the Ca-ions is also expressed by a simple and important relation,

$$Ca^{\cdot\cdot} \times t = \text{const.}$$

Milk, c.c.	$CaCl_2$, ‰	Time t for amount of rennet.			Constant, $Ca^{\cdot\cdot} \times t$ for amount of rennet.		
		1·0	0·5	0·25	1·0	0·5	0·25
8·0	—	95	48	24	57·0	28·8	14·4
8·0	0·05	88·6	45·6	23	57·6	29·6	15·0
8·0	0·1	79	41·6	22	55·3	29·1	15·4
8·0	0·2	66·4	36	19	53·1	28·8	15·2
8·0	0·5	48	26·4	14	52·8	29·0	15·4
8·0	1·0	30	18·2	10·6	48·0	29·1	17·0
8·0	2·0	17	11	6·8	44·2	28·6	17·7
8·0	5·0	10	7·4	5·4	56·0	41·4	30·2
8·0	10·0	13	9·2	6·2	137·8	97·5	65·7
8·0	20·0	22	15	8·6	453·2	309·0	177·2

The values of the constant, which show little change up to about 0·02% of calcium, are calculated on the assumption that the Ca-ions in the milk amount to 0·6‰; about 15% of the total calcium of the milk must then be in an ionised condition.

Further, as was shown in a subsequent paper (Hofm. Beitr., 1906, **8**, 15) the rennet-action a is, in general (not merely at the clotting point), directly proportional to the quantity of enzyme L and to the time t, that is

$$a = L.t. \text{ const.}$$

The more recent assertion of H. Köttlitz (Arch. intern. de Physiol., 1907, **5**, 140) that Schütz's rule holds also for rennetic action in no way diminishes the importance of Reichel and Spiro's results.

Several attempts have been made to estimate the value of the time-laws determined, on the one hand, for the action of rennet and, on the other, for that of pepsin, as criteria for the disputed identity of chymosin and pepsin.

The discussion of this question would not be in place in a chapter dealing with the chemical dynamics of the enzymes; but it may at least be asserted that, especially since Hammarsten's comprehensive investigation (H., 1908, **56**, 18), it is no longer possible to regard the clotting of milk simply as a peptic action. The influence of concentration, and also that of temperature, are so divergent in the cases of clotting and digestion, that it must at the least be assumed that two different enzymic groups are united in one molecule. This assumption, so long as the preparation of the enzymes in a pure state is not achieved, can be neither refuted nor proved, and is indeed, under present circumstances, of subordinate importance.

FIBRIN-FERMENT

E. Fuld (Hofm. Beitr., 1902, **2**, 514) mixed the plasma of bird-blood with the enzyme solution (obtained by extracting muscle with 0·8% sodium chloride solution). The velocity of clotting increased more slowly than the concentration of the enzyme, the results agreeing approximately with Schütz's rule but more accurately with the expression:

$$\frac{v_1}{v_2} = \left(\frac{E_1}{E_2}\right)^{0\cdot585},$$

where v_1 and v_2 are the velocities of clotting and E_1 and E_2 the concentrations of the enzyme. For protracted clotting periods, that is, for low enzyme-concentrations, this relation fails, the duration of clotting then showing disproportionate increase.

It has been shown, especially by Martin (Journ. of Physiol., 1905, **32**, 207), that the rule obeyed by chymosin and numerous other enzymes—that the same amount of action occurs for equal

values of the product $E.t$, no matter what the values of E and t—holds also for the fibrin-ferment.

ZYMASE

Although the " zymase " of pressed yeast-juice is accompanied by an extremely large quantity of various other substances and is further removed from a state of purity than is the case with any other enzyme, yet in recent years, especially by the work of Harden and Young, important information has been obtained concerning the chemistry of alcoholic fermentation.

If the final products, alcohol and carbon dioxide, of pressed yeast-juice containing sugar are studied quantitatively, as has been done by the author (H., 1905, **44**, 53), the following results are obtained:

The expression $\frac{1}{t} \cdot \log \frac{a}{a-x} = k$ gives moderately constant numbers during the first half of the reaction, but subsequently the value of k exhibits considerable diminution. To this effect, two principal causes contribute, firstly, the separation of protein substances which carry down part of the fermentation enzymes, and secondly, alteration of one of the activators of the zymase; this activator—consisting of organic compounds of phosphoric acid—is attacked by the lipase of the yeast-juice, the latter gradually becoming impoverished in the " co-enzyme " which is of such great importance to alcoholic fermentation.

The quantity of carbon dioxide evolved per unit of time was determined partly by the loss in weight of the solution and partly volumetrically.

4 grms. glucose in 20 c.c. of yeast-juice.

Minutes.	x (grms. CO_2).	$(a-x)$.	$k.10^4$.
0	—	1·800	—
80	0·078	1·722	2·4
315	0·299	1·501	2·51
379	0·360	1·440	2·56
505	0·460	1·340	2·54
1024	0·779	1·021	2·41
1180	0·810	0·990	(2·20)
1544	0·899	0·901	(1·95)
2119	0·955	0·845	(1·55)

For about 18 hours, the velocity of reaction is here moderately constant. This is, however, a favourable case and under other conditions, as is shown by the following table, the pressed juice remains unchanged only for 6–8 hours.

The influence of the concentration of the substrate is also shown by the following results.

20 c.c. juice+8 grms. of glucose in 20 c.c. of solution.

Minutes.	x(grms.)	$(a-x)$.	$k.10^5$.	Minutes.	x(grms.)	$(a-x)$.	$k.10^5$.
		3·909				3·909	
0		3·900	—	0		3·900	—
161	0·078	3·822	5·44	160	0·0815	3·8185	5·38
260	0·120	3·780	5·24	257	0·1195	3·7805	5·24
358	0·161	3·739	5·11	355	0·159	3·741	5·05
404	0·181	3·719	5·10	404	0·180	3·720	5·10
			5·21				5·19

20 c.c. juice+2 grms. of glucose in 20 c.c. of solution.

Minutes.	x(grms.)	$(a-x)$.	$k.10^4$.	Minutes.	x(grms.)	$(a-x)$.	$k.10^4$.
		0·977				0·977	
0	0	0·968	—	0	0	0·962	—
167	0·0855	0·8825	2·40	153	0·081	0·881	2·50
240	0·123	0·845	2·46	260	0·123	0·839	2·27
332	0·152	0·816	2·29	331	0·182	0·780	2·75
			2·38				2·51

In addition to these series of experiments, three others were carried out, also with concentrations of sugar in the ratio 4 : 1. The constants obtained were as follows:

	$k_1.10^5 : k_2.10^5$	
No. 4.	5·2 : 24·4	= 1 : 4·7
" 5.	2·5 : 12·0	= 1 : 4·8
" 7.	15·0 : 75·0	= 1 : 5·0
" 6.	20·0 : 97	= 1 : 4·85

It will be seen that the velocities are not, as they should be according to theory, independent of the concentration of the substrate; also they are not inversely proportional to the initial

concentration a, so that ka is not a constant magnitude but, in the region of concentration investigated, increases as a decreases. In each of the four series of experiments, $a_1 : a_2 = 4 : 1$.

	$k_1a_1 . 10^6$	$k_2a_2 . 10^6$
No. 4.	208	244
" 5.	100	120
" 7.	600	750
" 6.	800	990

Whilst, in general, the velocity of reaction increases either proportionally to, or slower than, the concentration of enzyme, the velocity of fermentation increases more rapidly than the concentration of the pressed juice but always slower than its square.

If the exponents n are calculated according to the equation

$$\frac{ln\ k_1 - ln\ k_2}{ln\ c_1 - ln\ c_2} = n,$$

it is found that, for a constant sugar-content, n increases with diminution of k, that is, with diminution of the concentration of the zymase:

$k_1 . 10^6$	n
100	1·29
86	1·33
35	1·52
12	1·67

The numbers appear to indicate that, with very high fermentative activity, proportionality between the concentration of the pressed juice and the velocity of fermentation is attained.

Finally, if pressed juice containing sugar is diluted, that is, the volume increased while the absolute amounts of the juice and sugar remain constant, the following relations are found:

(*a*)

Concentration	32 : 52 = 1 : 1·63
Velocity	192 : 282 = 1 : 1·47

(*b*)

	20 : 30 : 50 = 1 : 1·5 : 2·5
	120 : 188 : 315 = 1 : 1·57 : 2·63

The mean of these two experiments indicates, within the limits of concentration employed, approximate proportionality between concentration and velocity.

Prior to these experiments with pressed yeast-juice, A b e r s o n Rec. Trav. Chim. Pays-Bas, 1903, **22**, 78) had studied the course of the alcoholic fermentation of glucose by living yeast-cells.

It must however be emphasised that A b e r s o n ' s observations were made polarimetrically, so that he measured the amount of sugar disappearing during the reaction. But new measurements made by E u l e r and J o h a n s s o n (H., 1912, **76**, 347) with living yeast show that the diminution in rotation of a fermenting sugar solution is by no means proportional to the evolution of carbon dioxide. This is indicated by the following numbers:

No.	Temp.	Concentration of Sugar.	Amt. of yeast in 25 c.c. solution	Time. Min.	CO_2 evolved.		Change in rotation.		Difference. *B-A*
					Grms.	Per cent. *A*	Degrees.	Per cent. *B*	
1	30°	10%	0·5	125	0·0936	7·7	5·37 − 4·67 = 0·70	13 1	5·4
2	30	10	0·5	181	0·1492	12·2	5·32 − 4·46 = 0·86	16 2	4·0
3	30	10	1	42	0·0644	5·3	5·33 − 4·80 = 0·53	10·0	4·7
4	30	10	1	63	0·1160	9·5	5·33 − 4·48 = 0·85	16·0	6·5
5	30	10	1	232	0·4918	40·3	5·33 − 2·84 = 2·49	47·0	6·7
6	30	10	1	406	0·7256	59·4	5·33 − 1·85 = 3·48	65·9	6·5

The cause of this difference has not been fully elucidated.

Owing to this circumstance, A b e r s o n ' s experiments give information concerning only the first phase of alcoholic fermentation, namely, the transformation of the sugar. The results of his numerous experiments do not correspond with the expected law, but the values given in the last column of the following table—which is taken from that given on p. 97 (l o c . c i t .)—are far more nearly constant.

Minutes.	Rotation of the glucose solution.	$k = \frac{1}{t} \cdot \log \frac{a}{a-x}$	$k_H = \frac{1}{t} \cdot \log \frac{a+x}{a-x}$	
0	34·1	—	—	Temperature, 29·3°.
31	33·0	45·9	90·0	Volume, 600 c.c.
91	30·9	47·0	90·0	Amount of yeast:
125	29·7	48·0	90·0	0 mins. : 0·288 grm.
213	26·7	50·6	90·0	dry yeast per 50 c.c.
306	23·5	51·2	91·0	514 mins. : 0·294
393	20·9	54·1	90·0	grm. dry yeast per
514	17·7	55·4	90·3	50 c.c.

In the fermentation of similar sugar solutions with varying quantities of yeast, A b e r s o n obtained proportionality between the velocity of reaction and the amount of yeast (l o c . c i t., p. 84):

Grms. yeast	60	20	Grms. yeast	25	15
k	271	93·4	k	165	104

S l a t o r (Journ. Chem. Soc., 1906, **89**, 128), who measured volumetrically the carbon dioxide evolved, also found proportionality between the velocity of fermentation and the quantity of yeast added. He showed likewise that the initial velocity of fermentation is independent—except with very dilute solutions— of the sugar-content.

The fermentation experiments of H e r z o g (H., 1902, **37**, 149) and G r i g o r i e w (H., 1904, **42**, 299) also refer to heterogeneous systems.

The course of fermentation with permanent yeast (which, however, contained glycogen) is shown by the following table from H e r z o g ' s paper.

Concentration, 1·136 grms. glucose ($a=1$) and 1·2 grms. zymase (permanent yeast) in 100 c.c. Temperature 24·5°.

t	$a-x$	$\frac{10^6}{t}\cdot\log\frac{a}{a-x}$	$\frac{10^6}{t}\cdot\log\frac{a+x}{a-x}$
120	0·961	144	141
240	0·931	129	133
1200	0·687	137	117
1440	0·627	140	118
1740	0·560	145	117
2690	0·403	146	111
3000	0·365	146	108
4140	0·271	137	99

The exponent n of the enzyme-concentration which shows the increase of the velocity of the reaction, is greater in the experiments with permanent yeast than in those with the pressed juice. The following table indicates the behaviour of living yeast, acetone-permanent yeast and yeast-juice from a dynamical point of view.

Reaction constant.	Living yeast.	Acetone permanent yeast.	Zymase solution (pressed juice).
$k = \frac{1}{t} \cdot \log \frac{a+x}{a-x}$		$\frac{1}{t} \cdot \log \frac{a}{a-x}$ or $\frac{1}{t} \cdot \log \frac{a+x}{a-x}$	$\frac{1}{t} \cdot \log \frac{a}{a-x}$ (loc. cit., p. 62)
Influence of the amount of yeast or concentration of zymase $n = \frac{\log k_1 - \log k_2}{\log c_1 - \log c_2}$	1	2 (Herzog)	1·67–1·29
Influence of the concentration of sugar, a	$k \cdot a$ increases with increase of a	$k.a$ increases with diminution of a, within the region of concentration examined.	

As has been already stated, Buchner and Meisenheimer arrived at the conclusion that zymase—using the word in its wider sense—is a mixture of at least two enzymes, namely, zymase in a restricted sense which decomposes fermentable hexoses into lactic acid, and lactacidase, which breaks down the lactic acid into alcohol and carbon dioxide. There has been a considerable amount of discussion concerning the constituent chemical processes which take part in the transformation of sugar into alcohol and carbonic acid. Since grave objections have been advanced to the intermediate formation of lactic acid (compare the critical review by Buchner and Meisenheimer in Landw. Jahrbücher, 1909, **38**, Ergänz.-Band 5, 265), nothing final can be said as regards the number and nature of the participating enzymes.

That a "co-enzyme" plays an important part in fermentation must now be considered an established fact. The same is the case with the observation that addition of phosphates to pressed yeast-juice containing glucose not only accelerates the fermentation but also increases the total fermenting power or the amounts of alcohol and carbon dioxide which can be formed from a given quantity of sugar by the pressed juice (Harden and Young, Proc. Roy. Soc., 1905, **77**, 405; 1906, **78**, 369; 1908, **80**, 299).

These investigators have studied quantitatively the acceleration of fermentation by phosphates. Of their results, which are

expressed in the form of tables and curves, the following may be cited (Proc. Roy. Soc., 1908, **80**, 307):

Time after addition, in minutes.	Carbon dioxide evolved in preceding 5 minutes, with n c.c. of 0·3 molar potassium phosphate solution added.		
	$n=0$ c.c.	$n=10$ c.c.	$n=15$ c.c.
5	4·0	11·1	7·7
10	3·2	16·0	9·7
15	4·2	20·2	12·1
20	3·6	22·4	16·1
25	4·3	17·4	18·4
30	3·6	6·6	19·4
35	4·3	4·6	20·4
40	3·2	4·7	16·7
45	—	4·5	12·7
50	—	4·2	6·0
55	—	4·1	4·0

It is evident that a marked optimum of phosphate-concentration exists, the velocity of fermentation diminishing if this is exceeded.

The results of another series of experiments are shown graphically in the curves given below (Fig. 6). The concentrations of the solutions were as follows:

Curve *A* : 25 c.c. pressed juice + 5 c.c. phosphate + 15 c.c. bicarbonate
" *B* : 25 c.c. " +10 c.c. " +10 c.c. "
" *C* : 25 c.c. " +15 c.c. " + 5 c.c. "
" *D* : 25 c.c. " +20 c.c. " + 0 c.c. "

" These results suggest that the phosphate is capable of forming two or more different unstable associations with the fermenting complex. One of these, formed with low concentrations of the phosphate, has the composition most favourable for the decomposition of sugar, whilst the others, formed with high concentrations of phosphate, contain more of the latter, probably associated in such a way with the fermenting complex as to render the latter partially or wholly incapable of effecting the decomposition of the sugar molecule."

The influence of arsenates and arsenites on the evolution of carbon dioxide produced by yeast-juice has also been studied

in detail by Harden and Young (Proc. Roy. Soc., *B*, 1911, **83**, 451).

"A striking feature of the effect of the addition of a phosphate to yeast-juice is that the marked acceleration only continues until an amount of carbon dioxide has been evolved which is chemically equivalent to the phosphate added. Moreover, at the close of this period of enhanced fermentation, the added phosphate is no longer present in a form precipitable by magnesium citrate mixture, but has become converted into a hexosephosphate.

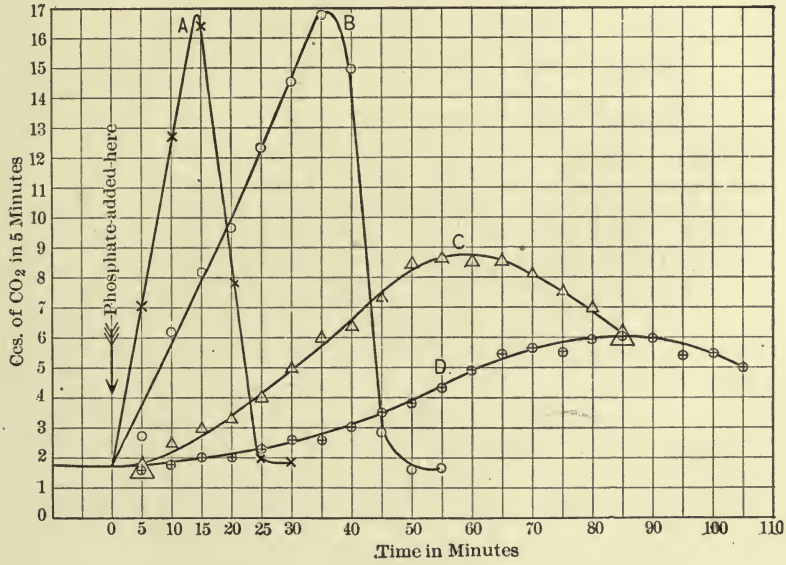


Fig. 6.

Neither of these phenomena occurs when an arsenate is substituted for the phosphate. The enhanced rate of fermentation continues long after an equivalent of carbon dioxide has been evolved and no organic combination of arsenic is formed.

The sharp contrast between the actions of arsenate and phosphate is clearly shown, when the effects of equivalent amounts of phosphate and arsenate on the same sample of yeast-juice are directly compared, as is done in the following experiments."

20 c.c. Yeast-juice

Time.	+5 c.c. 0·3 mol. phosphate.		+5 c.c. 0·3 mol. arsenate.		+0·75 c.c. 0·3 mol. arsenate	
	Total	Rate per 5 minutes.	Total.	Rate per 5 minutes.	Total.	Rate per 5 minutes.
5	7·1	7·1	11 4	11·4	21·7	21·7
10	19·8	12·7	26·5	15·1	46	24·3
15	36 1	16·3	43	16·5	71	25
20	43·8	7·7	59	16	95·3	24·3
25	45·7	1·9	75	16·3	119·8	24·5
30	47·5	1·8	—	—	—	—

Formation of the Phosphoric Ester

Contrary to the opinion of Iwanoff (H., 1907, **50**, 281; Centralbl. f. Bakt., 1909, II, **24**, 1), Harden and Young (Centralbl. f. Bakt., 1911, II, **26**, 178) regard the formation of the ester as due, not to a special enzyme, but to the zymase. According to Euler and Kullberg's and Euler and Ohlsén's experiments with the extract of dried yeast (H., 1911, **74**, 15, 1912, **76**, 468), the enzymic formation of the phosphoric ester can, however, be separated from the other fermentative processes, and the author (H., 1911, **74**, 13) hence suggests the name phosphatese for this synthesising enzyme.

If glucose is fermented by pressed yeast-juice in presence of phosphates, the latter very soon become combined organically. This also occurs with extract of Munich yeast, no matter whether this be dried at 40° or, as von Lebedew suggests, at 25–35°; in both cases, as was described by Harden and Young, fermentation and formation of phosphate proceed together.

But if another race of yeast with a lower fermenting power is dried at 40° in a vacuum and then extracted in the usual manner, no formation of phosphoric ester occurs with glucose (or mannose or fructose) (Table *a*, below). If, however, the glucose is partially fermented beforehand in absence of living yeast, organic combination of the phosphate takes place (Table *b*) (Euler and Ohlsén, Biochem. Z., 1911, **36**, 313).

TABLE *a*.

25 c.c. yeast-extract.
20 c.c. glucose solution (20%).
10 c.c. 5% Na_2HPO_4.

Minutes.	Grms. of $Mg_2P_2O_7$ from 10 c.c. of the mixture.
0	0·0399
150	0·0394
269	0·0394

TABLE *b*.

25 c.c. yeast-extract.
20 c.c. fermenting glucose solution (20%).
10 c.c. 5% Na_2HPO_4.

Minutes.	Grms. of $Mg_2P_2O_7$ from 10 c.c. of the mixture.
0	0·0414
74	0·0134
264	0·0000

Like other enzymes, the phosphatese may be precipitated from the aqueous yeast-extract by means of alcohol, a considerable proportion of its activity remaining after this treatment.

It is remarkable that the synthetic enzymic action of the extract is greatly increased by heating it at 40° for half an hour.

CATALASES

The decomposition of hydrogen peroxide effected by the action of unknown constituents—termed, after O. Loew, catalases—of the animal and vegetable body, is so easily followed quantitatively, that this process has formed the subject of a large number of investigations and is to-day one of the best-known enzymic changes. Especially from the fatty tissues of animals can solutions be prepared which, beyond their ability to decompose hydrogen peroxide, exhibit few enzymic activities; particularly small are the amounts of organic matter present in these liquids, which must therefore be comparatively pure. Also blood-serum, the organs of plants, etc., by precipitation with alcohol and suitable treatment of the precipitates, yield preparations which, per unit of weight, rapidly decompose hydrogen peroxide (O. Loew, Rep. U. S. Dep. of Agric., 1901, No. **68**; Senter, Zeitschr. f. physikal. Chem., 1903, **44**, 257 and Proc. Roy. Soc., 1904, **74**, 201; E. J. Lesser, Zeitschr. f. Biol., 1906, **48**, 1; L. van Italie, Soc. Biol., 1906, **60**, 150; Kastle and Loevenhart, Amer. Chem. Journ., 1903, **29**, 563; L. von Liebermann, Pflüg. Arch., 1903, **104**, 176 and Chem. Ber., 1905, **38**, 1524).

Catalase follows the theoretically simplest formulæ and relations with an approximation shown with few other enzymes,

possibly because the simple nature of the chemical process conditions especially simple relations, or possibly owing to the exclusion of any considerable complication by the relatively high degree of purity of the enzyme. The most important general conclusions arrived at are as follows:

The enzymic decomposition of hydrogen peroxide in neutral or acid solution is a reaction of the first order.

In dilute solutions of the peroxide, decomposition follows the law of mass action exactly. Numerous physico-chemical measurements of the action of catalases have been carried out, the following series of numbers being given by G. Senter and by H. Euler:

Blood-catalase. Senter, loc. cit., p. 282. $\frac{1}{106}$-molar H_2O_2-solution.				Fungus-catalase. Euler, Hofm. Beitr., 1905, **7**, 1. $\frac{1}{20}$-molar H_2O_2-solution.			
Minutes.	$a-x$	$k.10^4$.	$T=0°$.	Minutes.	$a-x$.	$k.10^4$.	$T=15°$.
0	46·1	—		0	8·0	—	
5	37·1	190		6	6·9	107	
10	29·8	192		12	5·8	116	
20	19·6	190		19	5·0	107	
30	12·3	193		55	2·5	100	
50	5	194					

Issaew (H., 1904, **42**, 102) also obtained very constant values of k with dilute solutions of hydrogen peroxide; with more concentrated solutions (0·01 N and above) the constants diminish.

Catalases are extremely sensitive—preparations of different origins to varying degrees—towards higher concentrations of hydrogen peroxide. Thus, blood-catalase is appreciably destroyed (perhaps oxidised) in about 0·01 N-H_2O_2, this action being naturally more rapid at higher than at low temperatures, the values of k being greatly diminished even at 30°. It is hence advisable to employ low temperatures in working with catalase. According to Waentig and Steche (H., 1911, **72**, 226), this injurious action is observable even at 0° in about N/80-hydrogen peroxide solution.

The reaction constants are, as they should be theoretically, nearly independent of the concentration of the peroxide, equal percentage amounts being decomposed per

unit of time by equal quantities of enzyme. Senter (loc. cit., p. 283) gives the following résumé:

$C_{H_2O_2}$	$k.10^4$	$C_{H_2O_2}$	$k.10^3$	$C_{H_2O_2}$	$k.10^3$
$\frac{1}{290}$ N	120	$\frac{1}{126}$ N	175	$\frac{1}{106}$ N	192
$\frac{1}{1100}$ N	122	$\frac{1}{460}$ N	188	$\frac{1}{440}$ N	225

(The two values of k in each of these pairs are comparable, one with the other, but not with those of the other pairs.)

The following results, given by Senter, indicate the influence of the concentration of the enzyme:

Concentrations, E, in the proportions	3	6	8	24
$k.10^4$	28	58	72	230
$k : C$	9·3	9·7	9·0	9·6

Within the limits of experimental error the velocities of reaction in very dilute ($\frac{1}{480}$-molar) solutions of hydrogen peroxide are therefore proportional to the concentrations of the enzyme. With stronger peroxide solutions, Senter found deviation from this rule. While $k.10^4$ is about 100 in weak catalase solutions, its value in presence of the threefold quantity of enzyme is approximately 360. That, under these conditions, the velocity of reaction increases more rapidly than the concentration of the enzyme, is confirmed by experiments made by Bach, who investigated the dynamics of the catalases with a preparation from beef-fat (Chem. Ber., 1905, **38**, 1878).

With N/210-peroxide solutions, Issaew observed deviation from proportionality in the opposite direction, that is, a slower increase of the velocity than of the enzyme-concentration. But the yeast-catalase he employed must, like all yeast-enzymes, have been very impure.

As was shown originally by Loew, catalases are extremely sensitive to acids. The action of acid has also been the subject of numerous quantitative investigations, which show that the enzyme is not permanently changed by acids, neutralisation of these being accompanied by return of the catalytic activity.

100 c.c. of catalase solution were poisoned, mixed with 100 c.c. of H_2O_2 solution and 25 c.c. then titrated; concentration of H_2O_2 in the mixture 0·005 –normal.

$\frac{1}{5000}$ norm. HCl.			$\frac{1}{10000}$ norm. HCl.			Without addition.		
Minutes	$C_{H_2O_2}$	$k.10^4$	Minutes	$C_{H_2O_2}$	$k.10^4$	Minutes	$C_{H_2O_2}$	$k.10^4$
0	27·9	—	0	27·9	—	0	27·9	—
$70\frac{1}{4}$	25·3	6	$15\frac{1}{4}$	25·6	26	$5\frac{1}{2}$	19·8	278
136	23·0	6	$35\frac{1}{2}$	22·4	27	15	10·5	275
195	20·9	6	$66\frac{1}{3}$	18·7	26	$25\frac{1}{6}$	5·8	258
1305	3·0	6	185	10·1	24			

$\frac{1}{10000}$-norm. HCl. Incubation period, 2 hours; then a small excess of NaOH added, and afterwards the H_2O_2.			Without addition.		
Minutes.	$C_{H_2O_2}$	$k.10^4$	Minutes.	$C_{H_2O_2}$	$k.10^4$
0	28·7	—	0	28·7	—
$5\frac{2}{3}$	23·8	121	5	25·2	120
$15\frac{1}{6}$	18·5	113	$19\frac{1}{2}$	16·9	121
$26\frac{1}{6}$	14·9	86	$31\frac{1}{3}$	11·9	127
$41\frac{1}{2}$	11·7	70	53	6·5	120

As is shown by the latter table, the catalase is not changed by the dilute hydrochloric acid solution, even after two hours. The incubation period, that is, the time during which the enzyme remains in contact with the acid, has been repeatedly shown to be without influence on the subsequent activity of the enzyme.

The catalytic power of catalases is also reduced by quite small amounts of baryta.

Temperature, 10°	$\frac{1}{500}$ norm. $Ba(OH)_2$.	$\frac{1}{500}$ norm. $Ba(OH)_2$.	Without
Catalase from fatty tissue	Incubation period,	Incubation period,	addition
(Euler, Hofm. Beitr.,	15 minutes.	40 minutes.	of baryta.
1905, 7, 12)	$k.10^3=40$	$k.10^3=40$	$k.10^3=60$

This sensitiveness towards alkali varies considerably with catalases of different origins.

A very slight increase of the alkalinity, however, appears to increase the velocity of the decomposition to some extent. Thus, the author found (loc. cit.) that the velocity of reaction of catalase from Boletus scaber is doubled by suspending pure magnesium hydroxide in the solution.

As is brought out most clearly by Sörensen's results (Biochem. Z., 1909, **21**, 131), the optimal activity of catalase is always exhibited in almost neutral solution.

The actions of acids and bases on this enzyme depend, in all probability, on the formation of salts by these electrolytes with the catalase.

Bach (Chem. Ber., 1905, **38**, 1878) has also investigated catalase quantitatively, his results in general agreeing with those required by theory.

OXYDASES

Substances of unknown constitution and composition, formed in the animal and vegetable kingdoms and capable of initiating oxidation changes, have been designated oxydases, generally without any definite proof of their mode of action; their sensitiveness to heat was, however, established and they were then classed with the enzymes. That these substances effected catalytic acceleration of oxidation processes was seldom, and could indeed only with difficulty be, proved, especially when isolated constituents of an organ or juice were not examined. On the other hand, many other enzymes are not catalysts in the strictest sense of the word, so that no limit could easily be drawn. The wide distribution of oxydases in vegetable and animal organisms renders it probable that these substances perform an important function in all life-processes; but what this function is, what reactions the oxydases bring about in the living animal or plant, still remains unknown. The members of this class of bodies which have as yet been obtained exhibit a somewhat limited sphere of action.

Aldehydases

Medwedew has made a very complete study of the oxidising agent of the liver, with reference to its action on salicylic aldehyde (Pflüg. Arch., 1896, **65**, 249; 1899, **74**, 193; 1900, **81**, 540 and 1904, **103**, 403).

As regards the final state or equilibrium, the following results were obtained. Case 1: relatively high concentration of salicylic aldehyde in neutral-acid solution. The concentration of the oxidation product (salicylic acid) is inversely proportional to the square-root of the amount of substance to be oxidised and approx-

imately proportional to the square of the concentration of the aldehydase.

Case 2: relatively high concentration of salicylic aldehyde in neutral-alkaline solution. One and the same quantity of the oxydase gives at the end of the reaction, that is, on complete exhaustion of the oxidising power, one and the same amount of acid, no matter what the concentration of the aldehyde.

In relation to the velocity it was found: (*a*) If to the quantity of oxydase m an excess of aldehyde a is added, the velocity of oxidation is proportional to the square-root of the concentration of aldehyde. In Medwedew's opinion, liver-oxydase is inactivated by the oxidation. But this must, in reality, only be a question of the consumption of an oxidising agent obtained from the liver.

(*b*) If the concentration of aldehyde is less than that which the oxydase present is able to oxidise, the velocity of oxidation is proportional to the square of the aldehyde-concentration, so that $dx : dt = k(a-x)^2$, where x is the concentration of the aldehyde changed up to time t and a the initial concentration.

How far these relations are quantitatively reproducible and hence are independent of the non-controllable composition of the liver-extract, and how far this interpretation of the numbers obtained is fitting, remain undecided. Doubts have, however, been expressed on these questions (compare Bach's remarks, Chem. Ber., 1905, **38**, 3791); but Dony-Hénault and van Duuren's experiments have led to different results (Bull. Acad. roy. Belgique, 1907, 577).

Slowtzoff's observation (H., 1900, **31**, 227) that "potato-laccase" oxidises paraphenylenediamine solution with a velocity proportional to the square-root of the quantity of this "laccase," also appears to the author to be insufficiently proved. What Slowtzoff investigated must have been a very impure mixture of a peroxydase and a substance allied to Medicago-laccase. The author obtained the latter component from potatoes by precipitation with alcohol. The nitrogen-content of the first precipitate amounted to 2·84%; after solution of the preparation, treatment with animal charcoal and further precipitation with alcohol, the proportion of nitrogen present fell to 1·6%, whilst the ability to accelerate the oxidation of hydroquinone solutions in presence of manganese salts remained undiminished.

As regards the "laccases," the investigations of Euler and Bolin (H., 1908, **57**, 80; 1909, **61**, 1 and 72) show that the oxydase of Rhus vernicifera and Rhus succedanea differs considerably from those of Medicago sativa, etc., which, according to Bertrand, are also termed laccases. As Bertrand himself showed, the Rhus-preparations are rich in manganese, whilst this is not the case with laccases of the Medicago-type. The former alone turn guaiaconic acid solutions blue directly and redden solutions of guaiacol. This effect of Rhus-laccase and also the power to transfer molecular oxygen to phenols (hydroquinone, pyrogallol) are destroyed by heating the solution to 100° for a short time, whilst Medicago-laccase is unchanged by heating.

In the case of Rhus-laccase, Bertrand (Bull. Soc. Chim., 1897, [iii], **17**, 619) established approximate proportionality between the manganese-content and the catalytic activity. The extraordinary sensitiveness of this preparation to acid is shown by the following measurements (Ann. Inst. Pasteur, 1907, **21**, 673).

The oxidation of guaiacol to tetraguaiacoquinone was measured. To a 2% guaiacol solution were added a little (0·1 grm. per litre) laccase (from Rhus succedanea) and sufficient sulphuric acid to give the mixture the acidity shown in the table. The numbers represent the intensities of the red colour—measured in a colorimeter—produced after 5 hours by the tetraguaiacoquinone formed.

Normality of the acid.	Series 1.	Series 2.
0	100	100
$\frac{1}{500,000}$	100	90·9
$\frac{1}{400,000}$	100	75·2
$\frac{1}{200,000}$	73·4	60·4
$\frac{1}{100,000}$	48·8	60·4
$\frac{1}{50,000}$	20·3	60·4

Oxydases of the Medicago-type, in presence of neutral manganese salts, accelerate the transference of molecular oxygen to the polyphenols.

A representation of the course followed by this action is given by the following experiments:

50 c.c. of a solution, 0·2-normal as regards hydroquinone and 0·001-equivalent normal as regards manganese acetate, and containing a weighed quantity of Medicago-oxydase, were shaken in a glass vessel from which the air had been replaced by pure oxygen. The oxidation of the hydroquinone to quinone or quinhydrone, produced by the manganese and oxydase, was measured by the diminution in the volume of oxygen in the vessel.

0·2 grm. oxydase per 50 c.c.		0·1 grm. oxydase per 50 c.c.	
Minutes.	Oxygen absorbed (c.c.).	Minutes.	Oxygen absorbed (c.c.).
5	1·3	5	1·0
10	2·3	10	1·7
15	3·0	15	2·2
20	4·1	20	2·5
30	5·9	30	3·1

It has been shown that Medicago-oxydase is a mixture of calcium salts of organic mono- and poly-basic hydroxy-acids, among which are glycollic, citric, malic and mesoxalic acids. The catalytic action of these pure salts corresponds closely with that of Medicago-oxydase, as is shown by the following numbers obtained under the conditions described above.

0·2 grm. calcium oxalate per 50 c.c.		0·1 grm. calcium glycollate 0·05 grm. calcium malate 0·05 grm. calcium mesoxalate } per 50 c.c.	
Minutes.	Oxygen absorbed (c.c.).	Minutes.	Oxygen absorbed (c.c.)
5	1·8	5	2·3
10	2·9	10	3·4
20	4·5	15	4·1
30	5·7	20	4·8
		30	5·9

As is well known, the oxidation of polyphenols, either alone or, more markedly, in presence of manganese salts, is considerably

increased by small quantities of alkali, so that especial care must be taken as regards the neutrality of the oxydase and of the calcium salts. In the above investigation they were absolutely neutral.

Since the salts of all the aliphatic hydroxy-acids appear to be more or less active in this direction, oxydases of this group must be of very frequent occurrence in the vegetable kingdom.

The indophenol reaction, which was first employed by Ehrlich in 1885 and consists in the formation of indophenol from α-naphthol and *p*-phenylenediamine, has been used recently by Vernon (Journ. of Physiol., 1911, **42**, 402) as the basis of a quantitative method. With the help of his method, this author has investigated quantitatively numerous oxidation phenomena induced by tissues.

According to the concentration of the substrate (naphthol and diamine), the amount of indophenol formed was found to be proportional either to the square of the quantity of enzyme, or to this quantity itself or to its square root.

PEROXYDASES

Presumably still more widespread and consequently of more general action are those substances of the animal and vegetable body which activate peroxides, including hydrogen peroxide, i.e., transfer the peroxide-oxygen to other substances; these are termed peroxydases.

The changes which the peroxydases themselves undergo, during this transference of oxygen from the peroxides to substances like α-guaiaconic acid, are unknown, and it has often been doubted whether the peroxydases should really be regarded as catalysts and as enzymes. Our conception of the latter, in particular, is so indefinite, that at the present time, when so little is known concerning the exact chemical nature of the peroxydases, discussion of this question is of little value.

Owing more especially to the work of J. Wolff and E. de Stoecklin (C. R., 1911, **153**, 139), substances such as potassium ferrithiocyanate, $K_3Fe(CNS)_6$, are however known which are not of organic origin and yet behave like the peroxydases.[1]

[1] It appears to be established that, in most biological oxidations which are regarded as enzymic, the true oxidising agent is peroxydic in character. This view was advanced almost simultaneously by Chodat and Bach

We shall proceed at once to a consideration of the numerical relations—due to Chodat and Bach and their pupils—characterising the action of these enzymes.

In the experiments which will first be described (Chem. Ber., 1904, **37**, 1342), definite quantities of peroxydase (from horseradish), hydrogen peroxide and pyrogallol were mixed in aqueous solution, the purpurogallin formed in 24 hours being collected on a tared filter, washed with 100 c.c. of water, and dried at 110° until of constant weight. Pyrogallol is not appreciably attacked by either peroxydase or hydrogen peroxide alone.

Three series of experiments were carried out:

A. With variation only of the amount of peroxydase.

B. With variation only of the amount of hydrogen peroxide.

C. With variation only of the amount of pyrogallol.

Temperature, 15–17°. Volume of the mixture, 35 c.c.

A

Hydrogen Peroxide, 0·10 grm.; Pyrogallol, 1 grm.

Amount of peroxydase, in grm.:	0·01	0·02	0·03	0·04	0·05	0·06	0·07	0·08	0·09	0·10
Purpurogallin formed, in grm.:	0·021	0·042	0·066	0·083	0·102	0·123	0·145	0·166	0·167	0·162

B

Peroxydase, 0·10 grm.; Pyrogallol, 1 grm.

Hydrogen peroxide, in grm.:	0·01	0·02	0·03	0·04	0·05	0·06	0·07	0·08	0·09	0·10
Purpurogallin, in grm.:	0·205	0·42	0·60	0·78	0·99	0·121	0·142	0·168	0·168	0·165

Experiments *A* show very clearly that with a constant amount (in excess) of hydrogen peroxide, the yields of purpurogallin are exactly proportional to the quantities of peroxydase employed; whilst, with varying amounts of hydrogen peroxide and a constant quantity (in excess) of peroxydase, the amount of change is, as experiments *B* show, proportional to the former.

An excess of peroxydase or of hydrogen peroxide is without influence on the oxidising capacity of the system peroxydase-hydrogen peroxide. From these results Bach and Chodat

(Chem. Ber., 1902, **35**, 1275, 2487, 3943; 1903, **36**, 600, 606, 1756) and by Kastle and Loevenhart (Amer. Chem. Journ., 1901, **26**, 539). The application of these noteworthy theories is often hindered by the uncertainty of their chemical foundations, so that the theoretical results need not be repeated here.

drew the conclusion that peroxydase and hydrogen peroxide take part in the reaction always in constant relations. They arrived thus at the hypothesis which had been previously advanced by Kastle and Loevenhart (Amer. Chem. Journ., 1901, **26**, 593), namely, that the peroxydase forms with the hydrogen peroxide a definite compound exhibiting more energetic oxidising properties than the peroxide alone. The result obtained by the two first-named investigators—that excess of hydrogen peroxide does not affect the change—indicates that the peroxydase is not used up during the course of the oxidation.

C

PEROXYDASE, 0·10 GRM.; HYDROGEN PEROXIDE, 0·10 GRM.

Pyrogallol, in grm.	1·5	2	3	4
Purpurogallin, in grm.	0·205	0·203	0·208	0·202

From the results of experiments *C* it is seen that the concentration of the pyrogallol is without influence on the magnitude of the change.

Since the formation of purpurogallin and the procedure described above are not suitable for the determinination of the time-law of peroxydase-action, Bach and Chodat (Chem. Ber., 1904, **37**, 2434) chose for this purpose the oxidation of hydrogen iodide.

Five c.c. of a solution, $\frac{1}{2000}$-normal with respect to acetic acid and hydrogen peroxide, were added to 45 c.c. of a solution containing $\frac{1}{2000}$-equivalent of potassium iodide and varying quantities of peroxydase. The iodine separated after a certain time was estimated by titration with thiosulphate.

$\frac{1\cdot25}{100{,}000}$-EQUIVALENT PEROXYDASE *

Minutes	1	2	4	6	8	10	12	20
c.c. thiosulphate	1·3	2·4	4·4	6·1	7·1	7·9	8·1	8·4

PARALLEL EXPERIMENT WITHOUT PEROXYDASE

Minutes	2	4	6	8	10	12	20
c.c. thiosulphate	0·25	0·45	0·6	0·8	0·9	1·1	1·6

$2\cdot5.10^{-5}$-EQUIVALENT PEROXYDASE

Minutes	1	2	4	6	8	10	20
c.c. thiosulphate	2·4	4·7	8·4	10·2	11·7	11·8	12·3

* The peroxydase-preparation was the same as in the above experiments. It activated exactly its own weight of hydrogen peroxide, or, according to Bach and Chodat's mode of expression, it had the activating power 1.

5.10^{-5}-EQUIVALENT PEROXYDASE

Minutes	1	2	4	6	8	10	20
c.c. thiosulphate	4·9	8·9	14·0	14·7	15·0	13·1	15·4

10.10^{-5}-EQUIVALENT PEROXYDASE

Minutes	1	2	4	6	8	10	20
c.c. thiosulphate	10·1	15·7	17·0	17·1	17·2	17·2	17·4

15.10^{-5}-EQUIVALENT PEROXYDASE

Minutes	1	2	4	6	8	10	20
c.c. thiosulphate	15·0	18·6	19·1	19·1	19·2	19·2	19·4

It will be seen first of all that, in the oxidation of hydriodic acid just as in that of pyrogallol, the peroxydase ultimately loses its activity, since after some time the procedure of the reaction becomes exactly that exhibited when no peroxydase is added; the rate at which the activity of the peroxydase diminishes increases with the concentration of the peroxide. Hence the results obtained with different concentrations of peroxydase are comparable only for those phases of the reaction where the enzyme still exerts almost its full activity

This is still the case at the end of the first minute, after which time the magnitudes of the change are exactly—within the limits of experimental error—proportional to the amounts of peroxydase:

Peroxydase-concentration $\times 10^5$	2·5	5	10	15
Amount of change after 1 minute (c.c.)	2·4	4·9	10·1	15·0

Comparison of the final states reached in the system hydriodic acid-peroxydase-hydrogen peroxide shows that the amount of the product of the reaction (iodine) is not, as with the oxidation of pyrogallol, directly proportional to the quantity of peroxydase, but increases more slowly than the latter. It is to be noted that the activating power of the peroxydase, $\frac{x}{a}$ (where x denotes the amount of hydrogen peroxide activated by a weight a of peroxydase), is considerably greater in the oxidation of hydriodic acid by hydrogen peroxide than in the oxidation of pyrogallol.

These experiments with hydriodic acid were extended by Bach (Chem. Ber., 1904, **37**, 3785), who determined the

separation of iodine occurring in 10 minutes at 22° in differently concentrated mixtures of HI, H_2O_2 and horseradish-peroxydase; his results are collected in the following table:

Increase of the change measured in c.c. of 0·01 N-thiosulphate.

	Peroxydase.	I. 12·5HI.	II. 25HI.	III. 37·5HI.	IV. 50HI.	V. 75 HI.	VI. 100HI.
A	1·25	3·1	4·4	5·0	5·4	5·3	5·4
B	2·5	5·1	7·1	7·9	8·5	8·6	8·5
C	5·0	7·0	9·5	11·3	13·2	13·6	13·6
D	10·0	9·3	12·9	15·7	17·9	21·4	25·1
E	15·0	10·9	15·9	18·5	21·6	26·0	29·3
F	21·0	10·7	17·6	21·4	25·5	29·1	33·6
G	25·0	10·8	17·8	24·0	28·4	32·6	37·1
Control	—	0·7	1·4	2·0	2·7	4·1	5·4

The increase in the amount of action in a given time rises both with the concentration of the peroxydase and with that of the hydriodic acid and, for each concentration of enzyme and acid, attains a certain limiting value and then remains constant; it must therefore be concluded that peroxydase, hydriodic acid and hydrogen peroxide react together in definite proportions.

It is readily seen from the preceding table that the product of the concentrations is (within certain limits) constant; for instance, $D \times I = C \times II$, etc.

Finally, comparison of the concentrations of hydriodic acid which correspond with different increments in the change shows that these increments are almost exactly proportional to the square-roots of the concentrations of the acid; this is evident from the following summary:

	I.	II.	III.	IV.	V.	VI.
Series D.....	9·2	12·9	15·7	17·9	21·4	25·1
Calculated.....	9·2	13·0	15·9	18·6	22·4	25·9
Series E.....	10·9	15·9	18·5	21·6	26·1	29.3
Calculated.....	10·9	15·3	18·8	21·8	26·5	30·7

Bach did not, however, obtain such regularity with another preparation, this giving a different relation, which had not previously been observed.

Increase of the change measured in c.c. 0·01 N-thiosulphate.

	Peroxydase.	I. 12·5HI.	II. 25HI.	III. 37·5HI.	IV. 50HI.	V. 75HI.	VI. 100HI.
A	1·25	1·2	2·4	3·3	4·2	4·2	4·1
B	2·50	2·2	4·2	6·1	8·1	8·3	8·2
C	5·0	3·6	6·0	9·4	12·1	15·2	15·8
D	10·0	4·4	8·3	12·2	14·6	20·7	25·9
E	15·0	5·0	9·6	13·8	18·5	27·4	36·6
F	20·0	5·0	10·1	15·1	20·1	30·2	41·0
G	25·0	5·1	10·2	15·6	20·4	30·0	40·8
Control	—	0·7	1·6	2·2	2·9	4·1	5·6

The results of series F and G show that, after the peroxydase-maximum is reached, the increase in the amount of transformation is exactly proportional to the concentration of the hydriodic acid.

For the complete utilisation of peroxydase, a definite acidity (concentration of the hydrogen-ions) of the liquid is necessary, the nature of the anion of the acid being without influence.

The darkening of many plant-juices owing to enzymic oxidation was found by Grafe and Weevers to be conditioned by the presence of catechol and this discovery has recently been confirmed by Miss Wheldale (Proc. Roy. Soc., *B*, 1911, **84**, 122). The study of the oxidation of catechol by plant-juices from a kinetic standpoint would be of great interest.

TYROSINASE

In conjunction with an investigation on melanotic pigments and the enzyme formation of melanins,[1] O. von Fürth and E. Jerusalem (Hofm. Beitr., 1907, **10**, 131) studied the mode of action of tyrosinase. The very complicated relations found by these authors led Bach (Chem. Ber., 1908, **41**, 216, 221), who regarded tyrosinase as a mixture of an oxygenase and a peroxydase, to establish the conditions of action of this enzyme.

The amounts of melanin formed were determined as follows: The juice was diluted tenfold with distilled water and 10 c.c. of this solution

[1] A step towards the elucidation of the chemistry of tyrosinase action has been made in a valuable investigation by Abderhalden and Guggenheim (H., 1907, **54**, 331).

mixed with 10 c.c. of tyrosine solution (containing 0·05% of tyrosine and 0·04% of sodium carbonate) and 30 c.c. of water; after 24 hours, the solution was acidified with 1 c.c. of 10% sulphuric acid and titrated with 0·002-normal permanganate until the latter was decolorised.

The tyrosinase was extracted from Russula delica.

1. Dependence of melanin-formation on the concentration of tyrosinase.

Into each of a series of eight beakers were placed 10 c.c. of the tyrosine solution and a certain quantity of the enzyme solution, the volume being then made up to 50 c.c. with water.

Permanganate solution used, in c.c.

	I.	II.	III.	IV.	V.	VI.	VII.	VIII.
Volume of enzyme solution (c.c.)	0·5	1·0	1·5	2·0	5·0	10·0	15·0	20·0
A. After 24 hours	10·8	14·2	17·3	19·8	25·8	30·4	33·6	35·8
B. After 48 hours	13·2	16·0	17·8	20·4	25·6	31·2	34·4	35·4

These results show that : (1) the amount of the product of the reaction increases with that of the enzyme, although more slowly than the latter, and (2) the rapidity with which the reaction comes to a standstill increases with the concentration of the enzyme. In these respects, tyrosinase behaves similarly to horse-radish-peroxydase (see p. 227).

2. Velocity of reaction and concentration of enzyme: Three 750 c.c. Erlenmeyer flasks were each charged with 100 c.c. of tyrosine solution, a certain volume of enzyme solution, and water up to a volume of 500 c.c.

	Volume of permanganate solution required.		
Quantity of enzyme solution per 500 c.c.	10 c.c.	20 c.c.	30 c.c.
	c.c.	c.c.	c.c.
Time in hours: 1	0·0	1·4	2·8
2	0·0	3·9	5·7
3	1·6	5·8	8·8
4	2·7	7·8	11·1
6	5·5	11·1	16·1
9	9·4	16·3	20·8
14	15·9	19·0	22·3
24	16·0	19·9	22·8

A reaction constant can scarcely be calculated with any degree of certainty, since the position of the end-value is doubtful. But if the times corresponding with equal amounts of change are compared, inverse proportionality between the amount of enzyme and the time of reaction is clearly evident: the product of the quantity of enzyme and the time is hence constant. That the initial and final stages of the reaction must be disregarded is explained by the slower initiation of the reaction with low than with higher concentrations of enzyme, whilst these higher concentrations lead to more rapid inactivation of the enzyme.

3. Velocity of reaction and concentration of substrate:

Three flasks, containing 25, 50 or 75 c.c. of the tyrosine and 30 c.c. of enzyme solution diluted to 500 c.c.

	Volume of permanganate solution required.		
Volume of tyrosine solution, in c.c.	25	50	75
After 1 hour	1·0	1·9	3·0
" 3 hours	3·1	6·3	9·2
" 5 "	4·8	9·4	13·9
" 8 "	7·0	12·4	14·7
" 24 "	8·4	12·6	16·2

With a constant concentration of enzyme, the quantity of melanin formed per unit of time is—apart from the final stages of the reaction—proportional to the amount of tyrosine present. Tyrosinase hence corresponds well with the law of mass action.

OXIDATION OF XANTHINE

In conclusion, it may be pointed out that Burian (H., 1905, **43**, 497) has investigated dynamically the oxidation of xanthine to uric acid. This reaction is, however, not one of pure oxidation, so that mention of this paper must suffice.

CHAPTER V

INFLUENCE OF TEMPERATURE AND RADIATION ON ENZYMIC REACTIONS

TEMPERATURE influences chemical systems in two ways:

It is, first of all, one of the factors which determine the position of equilibrium between the substances taking part in a reversible reaction. The degree to which the equilibrium changes with the temperature in any case is closely related to the heat-change of the reaction. If the equilibrium constant of a reaction is indicated, as before, by K, while U denotes the total heat-change determined calorimetrically, T the absolute temperature and R the gas-constant, van't Hoff's fundamental thermochemical law states that:

$$\frac{d\,lnK}{dT} = -\frac{U}{R.T^2}. \quad \ldots\ldots \quad (22)$$

If, therefore, the heat-change accompanying any reaction is small—and this is the case with most enzymic processes—the equilibrium is only slightly dependent on the temperature.

A much greater alteration with temperature is shown by the velocity with which a system proceeds towards its equilibrium or final position. In most cases, a rise of temperature of 10° doubles or trebles this velocity—a phenomenon to which van't Hoff first directed attention. With non-enzymic reactions, indeed, still higher temperature-coefficients are observed. From the abundant experimental data, the following figures, referring to reactions of biological interest, may be quoted:

Author.	Reaction.	Temp. interval.	$\frac{k_{t+10}}{k_t}$	μ
Price, Svenska Vet. Akad. Förh, 1899.	Ethyl acetate $+H_2O$	28–50°	2·4	17,390
Euler, Chem. Ber., 1905, **38**, 2551.	Formaldehyde $+NaOH$	50–85°	3·6	24,900
Spohr, Z. physikal. Chem., 1888, **2**, 194.	Inversion of cane-sugar	25–55°	3·6	25,600

The values of the quotient, $\frac{k_{t+10}}{k_t}$, hold only for a certain interval of temperature, the increase of the velocity of reaction per degree diminishing with rise of temperature. On the other hand, the constant μ retains its value over a very wide range of temperature. This constant is given by the formula derived theoretically by Arrhenius and found to be generally valid:

$$k_2 = k_1 e^{\frac{\mu}{R}\left(\frac{T_2 - T_1}{T_1 T_2}\right)}, \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (23);$$

it represents therefore an exact expression for the dependence of the velocity of reaction on the temperature. In this equation, k_1 and k_2 denote the reaction-constants at the absolute temperatures T_1 and T_2, while R is the constant of the gas-laws and e the base of the natural system of logarithms.

An influence as great as on the velocity of chemical reactions is exerted by the temperature on the vapour pressure of liquids and on the equilibria of certain dissociations. In the latter case this is explainable on the assumption that organic reactions are also effected by ions. The constant μ gives the heat of transformation accompanying the conversion of the participating molecules from the "normal" into the "active" state, and hence corresponds with the sum of the heats of dissociation of the substances taking part. If, for instance, the inversion of cane-sugar is considered, μ is the sum of the heat of dissociation of water, $U_{1\text{diss.}}$ and that $U_{2\text{diss.}}$, of cane-sugar (the latter taken as a base). The heat of dissociation of water is 13,450 Cals.; the value of $U_{2\text{diss.}}$ is still unknown, but it is known that with such extremely weak electrolytes as cane-sugar must be, the heats of dissociation are, in general, approximately of the same magnitude as that of water. On this assumption for $U_{2\text{diss.}}$, μ would be 26,900, while calculation of the experimental results according to Arrhenius's formula leads to the value 25,600 (Euler, Zeitschr. f. physikal. Chem., 1904, **47**, 353). The influence of temperature on the compound, substrate-catalyst (i.e., the hydrochloride, sulphate, etc., of the cane-sugar) is here apparently neglected. But, since the dissociation of strong acids and of salts changes comparatively slightly with the temperature, this influence is determined principally by the heats of dissociation of the cane-sugar and the water.

On the velocity of enzyme reactions, temperature has a twofold influence: that just referred to and, in addition, an action on the activity of the catalysing enzyme, which becomes more rapidly destroyed or permanently inactivated as the temperature rises.

The processes resulting from these two actions were first treated, theoretically and experimentally, by Tammann (Zeitschr. f. physikal. Chem., 1895, **18**, 426).

The simplest assumption is that the enzyme is inactivated in aqueous solution by a unimolecular reaction, independently of whatever else is in the solution. So that, if E is the initial concentration of the enzyme and y the concentration at time t, then:

$$\frac{dy}{dt}=k_E(E-y),$$

hence

$$E-y=E.e^{-k_E^t}, \quad . \quad . \quad , \quad . \quad . \quad . \quad . \quad (24)$$

where e is the base of the natural system of logarithms, and

$$k_E=\frac{1}{t}ln\frac{E}{E-y}. \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (25)$$

If further, a denotes as usual the original concentration of the substrate, which undergoes decomposition according to an equation of the first order, it follows that

$$v=\frac{dx}{dt}=k(a-x)(E-y), \quad . \quad . \quad . \quad . \quad . \quad (26)$$

that is, the velocity with which the reaction proceeds at time t must be equal to the product of the amounts of enzyme and substrate still present.

Substitution in the last equation of the value of $E-y$ from Eq. (24) gives, as Tammann showed, the integral equation:

$$ln\frac{a-x}{a}=-\frac{k}{k_E}\cdot E(1-e^{-k_E^t}). \quad . \quad . \quad . \quad . \quad . \quad (27)$$

From Eq. (27), Tammann calculated the "false equilibrium," that is, the final state of the enzymic reaction, and compared the result with that determined experimentally with the system emulsin-salicin. The following tables are taken from those given by Tammann.

To solutions of salicin, previously heated, were added varying quantities of emulsin, 100 c.c. of each of the mixtures containing 3·007 grms. of salicin and the amount of enzyme shown in the table.

PERCENTAGE OF SALICIN HYDROLYSED

Emulsin (grm.).	72 hours.	104 hours.	148 hours.	
0·250	63·4	65·1	65·4	Temp., 65°
0·125	48·3	50·2	50·4	
0·0312	16·4	17·0	16·8	

Emulsin (grm.).	45 hours.	86 hours.	166 hours.	
0·250	(101·2)	99·2	—	Temp., 45°
0·125	97·5	97·5	(100·2)	
0·0156	59·3	65·7	67·6	

Emulsin (grm.).	45 hours.	93 hours.	334 hours.	
0·250	96·5	98·0	100.2	Temp., 26°
0·125	96·5	97·5	99·6	
0·0156	85·8	92·1	98·0	

As will be seen, the higher the temperature, the sooner the reaction comes to a standstill, i.e., the more rapidly the enzyme is inactivated.

It must particularly be pointed out that a distinction is to be made between the permanent non-reversible inactivation which every enzyme undergoes, especially at higher temperatures, and the inactivation due to the products of reaction and disappearing when these are removed. In the latter case, where the catalysing enzyme is held by the reaction-products, the reaction retards itself by consuming its own catalyst. Such catalytic retardation has been treated by Ostwald (Lehrbuch, II, **2**, 271).

The differential equation for this case,

$$\frac{dx}{dt} = (k_1 - k_2 x)(A - x)$$

gives, on integration,

$$\frac{1}{k_1 - Ak_2} ln \frac{Ak_2(k_1 - k_2x)}{k_1(Ak_2 - k_2x)} = t. \quad . \quad . \quad . \quad . \quad (28)$$

As Ostwald pointed out, k_2x at the beginning of the reaction must not be greater than k_1, as otherwise the reaction does not take place. The reaction therefore leads here to a " false equilibrium " or end-state.

The influence of rise of temperature on an enzymic reaction is hence two-fold: 1, Acceleration of the reaction and, 2, inactivation of the enzyme. So that both k and k_E increase as the temperature rises, and when the influence of temperature on an enzymic reaction is to be defined, the temperature-coefficient should be given—as was done by Tammann —both for k and for k_E. The temperature-function of the two magnitudes is best expressed by Arrhenius's formula.

Owing to the dependence of the stability of the catalyst on the temperature, the temperature-curves of enzymic reactions differ in appearance from those of most other chemical processes. Thus, they show an optimum, the temperature-coefficient at a certain temperature being zero, owing to the increased velocity of decomposition of the substrate being exactly compensated by the increased rate of destruction of the enzyme; further rise of temperature is then accompanied by decrease of the velocity of reaction.

It was assumed on p. 233 that the decomposition of enzymes obeys the formula for unimolecular reactions.[1] This is best ascertained by keeping the enzyme for a certain time in aqueous solution at the temperature to be investigated, then mixing it—where possible at a lower temperature—with the substrate and calculating the constant k_E from the initial velocity. The values of k_E obtained at different temperatures then give the constant μ of the temperature-formula of Arrhenius given on p. 232,

$$k_2 = k_1 e^{\frac{\mu}{R} \cdot \frac{(T_2 - T_1)}{T_2 T_1}}.$$

[1] Whether all enzymes decompose according to this law is still questionable. According to Senter it is not the case with catalase.

Spontaneous decomposition of:	μ	
Emulsin in 0·5% solution	45,000	Tammann
Rennet in 2% solution [1]	90,000	Madsen and Walbum [3]
Trypsin in 2% solution	62,034	Madsen and Walbum [3]
Pepsin in 2% solution [2]	75,600	Madsen and Walbum [3]
Yeast-invertase	72,000	Euler and af Ugglas
Dry emulsin	26,300	Tammann

For yeast-invertase, Euler and Kullberg (H., 1911, **71**, 134) have shown that μ is independent of the impurities arising from the yeast and hence represents a well-defined constant.

Mention must also be made of the measurements made by Nicloux with lipase (Soc. Biol., 1904, **56**, 701, 839, 868), the value obtained for μ being about 26,000.

Senter (Zeitschr. f. physikal. Chem., 1903, **44**, 257) states that the destruction of blood-catalase at 55° is about 6–7 times as rapid as at 45°; μ is about 50,000.

Special emphasis must be laid on the high values of μ compared with those of the corresponding constants for other reactions, such as those given on p. 231. The result obtained with dry emulsin shows the slight sensitiveness to heat of dry enzymes relatively to that shown in the dissolved state. Similar observations of a qualitative character have often been made with other enzymes. The numerical values obtained under these conditions for μ are, as is easily understood, dependent in a high degree on

[1] Merely by shaking for a few minutes, even at room-temperature, enzyme solutions are rapidly inactivated. This action was observed almost simultaneously by Signe and Sigval Schmidt-Nielsen with rennet, Abderhalden and Guggenheim with tyrosinase, and Shaklee and Meltzer with pepsin.

As was formerly assumed by the author, inactivation by shaking or denaturation and skin-formation are due partly to a surface-action and partly to the influence of atmospheric oxygen; with many colloidal solutions, shaking in the air produces flocculation (S. and S. Schmidt-Nielsen, H., 1910, **68**, 317).

[2] According to Shaklee (Zentralbl. f. Physiol., 1909, **23**, 4), at 37° pepsin loses its activity according to the formula for bimolecular reactions

$$k = \frac{1}{at} \cdot \frac{x}{a - x},$$

where a is the original amount of pepsin and x the amount changed (destroyed) in time t. After 12 days, 86% of the enzyme is destroyed.

[3] Calculated by Arrhenius (Immunochemistry, p. 98).

the previous treatment and especially on the moisture-content of the enzyme-preparations, and they have a real significance only if the compositions of the preparations are suitably defined.

The velocity of decomposition of dissolved enzymes and its temperature-coefficient are also largely dependent on the other substances present in the solution. Small proportions of acids or bases often influence the stability enormously; thus, for example, bases occasion a very considerable acceleration of the destruction of rennet and of trypsin (Arrhenius, Immunochemistry, p. 88).

A very complete study of the influence of acids and alkalies on the destruction of invertase has been made by Hudson and Paine (Journ. Amer. Chem. Soc., 1910, **32**, 985), from whose paper the following figure (p. 238) is taken.

An extract from Table 2 of Hudson and Paine's paper shows that at 0–45°, the temperature-coefficient of the destruction by acid or alkali does not differ from that of ordinary reactions. This is very remarkable, for, at the optimal stability ($H^{\cdot}$ = about 10^{-5}), the temperature-coefficient of enzyme-destruction is, at any rate between 55 and 65°, extremely high. It recalls the temperature-coefficients of the denaturation of proteins recently measured by Martin, and it is to be supposed that with the enzymes it is a case of similar denaturation, i.e., of a change of their state of solution. In this connection, an ultramicroscopical investigation would be of interest.

In many cases, salts produced a marked increase in the stability of enzymes; thus, according to Vernon (Journ. of Physiol., 1901, **27**, 174), the optimum of pancreas-diastase in a starch solution containing 0·2% of sodium chloride is at 50°, whilst in pure aqueous starch solution the optimum temperature is 35°.

Apart from this, it is known that many neutral substances, particularly proteins [1] and other colloids (Journ. of Physiol., 1904, **31**, 346), but more especially the specific substrates and reaction-products, increase the stability of the enzymes to a marked extent. This latter fact was pointed out by O'Sullivan and Tompson (loc. cit.). Biernacki (Zeitschr. f. Biol., 1891, **28**, 49) and Vernon (Journ. of Physiol., 1901, **27**, 269; 1902, **28**, 375, 448; 1904, **31**, 346) made the same observation in the

[1] Non-specifically hydrolysable proteins often act as "buffers."

case of trypsin,[1] and W o h l and G l i m m (Biochem. Z., 1910, **27**, 365) in that of amylase. In other instances, for example, according to the author's measurements, with invertase, the protective action of the substrate is slight. The stability of this enzyme is, however, considerably increased by the presence

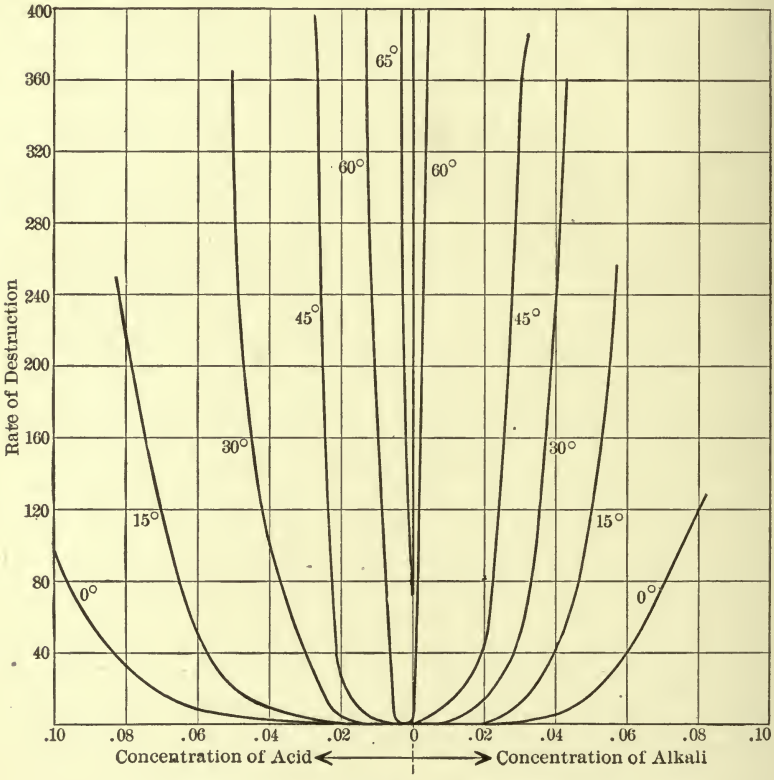


Fig. 7.

of fructose (H u d s o n and P a i n e, Journ. Amer. Chem. Soc., 1911, **32**, 988).

[1] In this connection mention must be made of the observations of W. C r a m e r and B e a r n (Proceedings of the Physiol. Soc., June 2, 1906; see Journ. of Physiol., 1906, **34**, XXXVI), according to whom active pepsin is retarded by the addition of pepsin solutions inactivated at 60°, whereas preparations inactivated at 100° produce little or no retardation.

In dilute solution diastase keeps better than in more concentrated ones (Effront, Enzymes and their Applications, 1902, p. 56).

The temperature-coefficients of the enzymic reactions themselves, that is, the alterations of the velocity-constants k with the temperature are of the same order of magnitude as those of other chemical processes. An attempt should always be made to measure the temperature-coefficient in a region of temperature where the destruction of the enzyme comes into consideration as little as possible. In most of the previous measurements, the distance from the optimum is so slight that the constants μ are influenced by the destruction of the enzyme and are consequently too low. Further, as the following examples show, the errors of observation are generally very large, even in the work of reliable experimenters.

Auld (Journ. Chem. Soc., 1908, **93**, 1275): measurements on amygdalin-emulsin.

$k_{t+10} : k_t$

$k_{25} : k_{15} = 2{\cdot}37$	$k_{45} : k_{35} = 1{\cdot}37$
$k_{30} : k_{20} = 1{\cdot}81$	$k_{50} : k_{40} = 1{\cdot}71$
$k_{35} : k_{25} = 2{\cdot}14$	$k_{55} : k_{45} = 1{\cdot}04$
$k_{40} : k_{30} = 1{\cdot}68$	

Kastle and Loevenhart (Amer. Chem. Journ., 1900, **24**, 501) left tubes containing 4 c.c. of water, 0·1 c.c. of toluene and 1 c.c. of a 10% liver- or pancreas-extract for 5 minutes in baths at 40°, 30°, 20°, 10°, 0° and −10°, so that they assumed these temperatures. Ethyl butyrate (0·26 c.c.) was then added and the solutions titrated after 30 minutes.

Temperature.	Percentage hydrolysed.	
	By liver-extract.	By pancreas-extract.
40°	11·29(?)	2·82
30	5·96	3·16
20	5·27	2·51
10	3·89	1·88
0	2·26	1·25
−10	0·70	

The value obtained with liver-extract at 40° must be due to an error of experiment. Hanriot (C. R., 1897, **124**, 778) obtained similar results with his esterases from serum and pancreas.

Reference must finally be made to a series of experiments made by Chodat (Arch. Sci. phys. nat., 1907, **23**, 13) on the action of tyrosinase on tyrosine.

The second row below gives the times in which the solution had attained a certain intensity of colour.

Temperature.....	0°	10°	20°	30°	40°	45°	50°
Time (minutes).....	180	100	60	40	30	20	10

Very unreliable quantitatively are the temperatures given by Tammann (loc. cit.) for invertase and cane-sugar, Lindner and Kröber (Chem. Ber., 1895, **28**, 1053) for maltase, Hanriot and Camus (C. R., 1897, **124**, 235) for serum-esterase and monobutyrin, Miquel (see Herzog, loc. cit.) for urease, and by experiments made according to Mett's method.

Consequently reference will only be made to the calculation of these results by Herzog (Zeitschr. f. allg. Physiol., 1904, **4**, 189).

In the following table (p. 241) are collected the data as yet obtained concerning the temperature-coefficients of enzyme-reactions.[1]

In other cases the value of μ changes considerably with temperature; this is shown, for instance, by Müller-Thurgau's results with amylase (Landw. Jahrb., 1885, 795).

An extended investigation of the temperature coefficients of alcoholic fermentation by living yeast has been made by Slator, with the following results:

t	$v_{t+10} : v_t$
5°	5·6
10	3·8
15	2·8
20	2·25
25	1·95
30	1·60

[1] Complicated biological processes, such as the assimilation of carbon by green leaves, are not considered here.

Author.	Substrate and enzyme.	Temp.-interval.	$\frac{k_{t+10}}{k_t}$	μ
Kastle and Loevenhart (loc. cit.)	Ethyl butyrate, esterase	20–30°	1·3	4,650
Tammann (loc. cit.)	Cane-sugar, invertase	20–30	1·4	6,000
Kjeldahl (Medd. fra Carlsberg Lab., 1881, 335)	Cane-sugar, invertase	30–40	1·5	7,800
O'Sullivan and Tompson (loc. cit.)	Cane-sugar, invertase	40–50	1·4	6,800
Senter (Zeitschr. f. physikal. Chem., 1903, **44**, 257)	H_2O_2, catalase	0-10	1·5	6,200
Chodat (loc. cit.)	Tyrosine, tyrosinase	20–30	1·5	7,200
Euler and af Ugglas (loc. cit.)	Cane-sugar, invertase	0–20	2·0	11,000
Vernon (Journ. of Physiol., 1901, 27, 190)	Starch, amylase	20–30	2·0	12,300
Vernon (ibid.)	Milk, rennet	20–30	2·1	13,400
Vernon (ibid. 1903, **30**, 364)	Witte's peptone, trypsin	15–25	2·3	14,300
Tammann (loc. cit.)	Salicin, emulsin	15–25	2·4	15,000
Vernon (Journ. of Physiol., 1903, 30, 364)	Witte's peptone, erepsin	15–25	2·6	16,400
Taylor (Journ. of Biol. Chem., 1906, 2, 87)	Triacetin, lipase	18–28	2·6	16,700
Fuld (Hofm. Beitr., 1908, 2, 169)	Milk, rennet	30–40	3·2	22,000
Bayliss (Arch. Sci. Biol., St. Petersburg, 1904, 11, 261, Supplement)	Casein, trypsin	20·7–30·7	5·3	37,500
Heterogeneous systems				
Aberson (Rec. Trav. Chim. Pays-Bas, 1903, 22, 100)	Sugar, living yeast	18–28°	2·7	15,600
Herzog (H., 1902, 37, 160)	Sugar, permanent yeast	15–25	2·8	18,000

In consequence of the foregoing data it must again be emphasised that the influence of temperature on enzymic reactions is exactly defined only by determination of the inactivation constant k_E at a given temperature and measurement of the initial velocities of the reaction itself at temperatures at least 20° lower than the above.

The following very instructive numbers, obtained by Gerber (Soc. Biol., 1903, **63**, 375), show how the temperature-coefficient of an enzymic reaction may depend on the quantity of enzyme present:

Concentration of rennet.	Time of coagulation of milk at different temperatures.						
	25°	30°	33°	36°	39°	42°	45°
0·005	30′ 20″	29′ 00″	no coag. in 360′	no coag. in 360′	no coag. in 360′	No coag. in 360′	No coag. in 360′
0·010	14 45	11 30	7′ 00″				
0·015	9 40	7 25	4 40	5′ 35″			
0·020	7 30	5 00	2 30	3 15	5′ 30″		
0·025	6 15	3 30	2 05	2 20	2 40		
0·030	4 40	2 50	1 40	1 30	1 40		
0·040	3 40	2 20	1 30	1 00	0 50		
0·050	3 00	1 50	1 10	0 55	0 40	2′ 05″	
0·075	2 20	1 20	0 50	0 40	0 30	1 00	
0·100	1 40	1 00	0 40	0 30	0 25	0 35	0′ 45″

Very striking is the result of a comparison between the values of μ for the acceleration of the inversion of cane-sugar by an acid and by invertase respectively; the data on pp. 231 and 241 give:

Cane-sugar-hydrochloric acid.	Cane-sugar-invertase.
$\mu = 25{,}600$	$\mu = 11{,}000$

Although enzymic temperature-coefficients had been determined by very reliable experimenters (Kjeldahl, Tammann, O'Sullivan and Tompson), new investigations on this point were to be desired. On this account, the author and Miss B. af Ugglas have made fresh determinations of these coefficients under various experimental conditions (H., 1910, **65**, 124); at the optimum concentration of hydrogen-ions, it was found that $k_{30} : k_{20} = 1{\cdot}9 - 2{\cdot}1$. This is in agreement with the result obtained by Visser (Zeitschr. f. physikal. Chem., 1905, **52**, 257), namely 2, for the ratio $k_{20} : k_{10}$.

The first conclusion that could be drawn is that the constant μ includes the heat-change occurring during the formation of the compound between the cane-sugar and the acid or invertase. It is also possible that the small rise of temperature taking place during enzymic inversion may be due to rise of temperature not only irreversibly destroying the invertase but also reversibly inactivating it; apart from the destroyed portion, the invertase resumes its original activity at the lower temperature. Hence, rise of temperature renders the substrates, cane-sugar

and water, more active and the catalysing enzyme less active. Other reactions, for instance, the hydrolysis of esters, also exhibit smaller temperature-coefficients for enzymic than for acid catalysis. For non-enzymic reactions, Slator (Zeitschr. f. physikal. Chem., 1903, **45**, 547) found temperature-coefficients varying with the catalyst.

Very ill-defined is the so-called "optimum temperature," the position of which depends entirely on the period or phase of the reaction considered. Indeed, even at the optimal temperature the enzyme undergoes partial destruction during the reaction, so that if comparison is made of the times taken for the reaction to proceed to the extent of one-half, the optimum is apparently lower than if only the first one-fifth of the reaction is considered. The real initial velocity will, in general, show no optimum if the time of observation is made short enough. For practical purposes it may be of interest to know the temperature at which the reaction proceeds most rapidly, and it would then be best to consider the times in which, say, 90–95% of the substrate is decomposed. In any case, in giving the optimum temperature, it must be stated for which stage of the reaction it holds.

Still more indefinite are most of the data on "maximum temperatures" (temperatures of destruction). Measurements of these temperatures are of value only when the duration of the experiment and the magnitude of the weakening are determined. It is therefore advisable to give that temperature at which the enzyme is weakened, for example, to the extent of one-half in 30 minutes; still better is it to measure the inactivation constant k_E of the dissolved enzyme at a given temperature.

The presence and concentration of other substances in the solution may influence both the optimal and the maximal temperatures very considerably; marked alterations of these temperatures are produced especially by acids and bases, so that in measurements of the stability it is necessary to define the concentration of the H·- or OH'-ions. Non-electrolytes—so long as they do not constitute the substrate or a product of the reaction —appear to have little influence, the stability-constant being, therefore, readily reproducible (cf. the measurements of B. af Ugglas and Kullberg, loc. cit.).

The very great differences existing, according to R. Huerre

(C. R., 1909, **148**, 300), between the maltase of white maize and that of yellow maize, may be due to the action of a foreign substance of some kind; but it can, by no means, be denied that the two sorts of maize may contain different maltases.

For most enzymes the optimal temperature is stated to be between 37 and 53°, and the maximal temperature between 60° and 75°.

Many oxydases resist surprisingly high degrees of temperature. That known as Medicago-laccase is especially stable to heat, as is to be expected from its composition (see p. 62). Other oxydases are destroyed only at 80–90° (Kastle, Chem. Zentralbl., 1906, **77**, i, 1554).

Also peroxydases, at any rate in the natural juices, are only slightly injured by heating. Thus, a preliminary experiment with the juice from pressed horseradish showed that heating for two hours at 60° diminished the activity of the peroxydase on guaiaconic acid only in the proportion of 7 : 5. For μ the very low value, 4000, was obtained.

Apparently still more resistant is myrosin, which, according to Guignard's experiments (C. R., 1890, **111**, 249, 920; Bull. Soc. Bot. de France, 1894, [3], 418), is not destroyed even at 81°, although a knowledge of the duration of the heating is to be desired.

Peculiar behaviour towards high temperatures has been observed by Delezenne, Mouton and Pozerski (Soc. Biol., 1906, **60**, 68, 390) in the case of papain. At temperatures up to 40°, papain exerts no digestive action on egg-albumin or blood-serum. Digestion only occurs, and then very rapidly, on further heating of the solution. These results were completely confirmed by Jonescu (Biochem. Z., 1906, **2**, 177), who studied the differences between ordinary and "heat-digestion," while Gerber (Soc. Biol., 1909, **66**, 227) has also remarked the notable resistance of papain to high temperatures.

Towards low temperatures, enzymes appear to be highly resistant; thus, Miss White showed that the enzymes of cereals are not destroyed by exposure to the temperature of liquid air for two days (Proc. Roy. Soc., *B.*, 1909, **81**, 440).

To sum up, the sensitiveness of enzymes to heat is, indeed, very great, but is not so marked as with the toxines, for which

Madsen found the temperature-constants of spontaneous decomposition in solution to be as high as $\mu = 198{,}500$.

Enzymic reactions appear to have rather lower temperature-coefficients than the corresponding non-enzymic catalyses.

INFLUENCE OF RADIATION

The manifold action exerted by light on the processes occurring in living cells and tissues has naturally given rise to the impression that enzymes are sensitive to rays of various wave-lengths. The success of the modern photo-therapeutic methods of Finsen and others, on the one hand, and the knowledge that toxines undergo very rapid destruction in the light, on the other, endow this subject with considerable practical and scientific interest.

1. Light-rays

It may be mentioned firstly that, in general, enzymes do not appear to exhibit so high a degree of sensitiveness to light as do the toxines. This observation was, indeed, made some years ago by O. Emmerling (Chem. Ber., 1901, **34**, 3811).

Hertel subjected a number of enzymes and toxines to the influence of light-rays and observed, among other results, that trypsin and also diastase and rennet are weakened by rays having the wave-length 280 $\mu\mu$. His investigations also showed that the destruction of enzymes requires a longer exposure than does that of the toxines, the enzymes being therefore decidedly more photo-stable substances than the toxines (Biol. Zentralbl., 1907, **27**, 510).

Just as in the study of the thermo-lability of the enzymes, so also in investigating the action of light, a distinction must be drawn between the destruction of the enzyme by light and the alteration of the enzymic action under the influence of the radiation. In so far as the results already obtained indicate, the former influence predominates, the observed retardations of enzyme-action being therefore due principally to a partial annihilation of the enzyme molecule.

In his paper cited above, Emmerling states that, in absence of air, invertase, lactase, emulsin, amylase, trypsin [1]

[1] According to Fermi and Pernossi (Zeitschr. f. Hygiene, 1894, **18**, 83), pepsin and trypsin are weakened in sunlight.

and pepsin are injured but slightly by daylight; yeast maltase and rennet are rather more sensitive to these conditions. The activity of the last-named enzyme, in 1% aqueous solution, was reduced to one-half by diffused sunlight, and to one-third by direct sunlight, in the course of five days.

Downes and Blunt found this action of light to be of slight extent and the same result was obtained by F. Weis (Medd. fra Carlsberg Lab., 1903, **5**, 135) with the proteolytic enzyme of malt and by Schmidt-Nielsen (Medd. fra Finsens med. Lysinstitut, 1903) with chymosin. On the other hand, F. G. Kohl (Beitr. z. Bot. Zentralbl., 1908, **23**, 64) states that invertase is considerably affected even by diffused daylight. The disagreement between this statement and the earlier observations is explained by the results of Jamada and Jodlbauer (Biochem. Z., 1908, **8**, 61), who showed that the rays of sunlight which pass through glass are alone capable of injuring invertase, but to a marked extent only in presence of oxygen.

With reference to the stability of catalase under the action of light, a comprehensive investigation was carried out by Lockemann, Thies and Wichern (H., 1909, **58**, 390). The inhibiting action of light on blood-catalase, both when the blood-solution is kept and during the reaction with hydrogen peroxide, is greatest with white and least with red light, blue occupying an intermediate position.[1] Also in this case, according to Zeller and Jodlbauer (Biochem. Z., 1908, **8**, 84), appreciable injury is produced by visible rays only when oxygen is present; a similar result was arrived at by Zeller and Jodlbauer, and almost simultaneously by Bach (Chem. Ber., 1908, **41**, 225), with peroxydase.

The results of a large number of investigations are in agreement in indicating that the inhibiting influence of ultra-violet rays is much greater than that of the visible rays. The action of these rays was investigated firstly and very com-

[1] In absence of catalase, additions of sodium chloride retard the decomposition of hydrogen peroxide by light. For the sensitiveness to light of solutions of the peroxide either containing or free from catalase, Wo. Ostwald (Biochem. Z., 1908, **10**, 1) found the influences of different kinds of light to be in the following order of diminishing magnitude: white, violet, yellow, dark.

pletely by Reynolds Green (Trans. Roy. Soc., 1897, **188**, 167), who showed that violet and ultra-violet rays destroy diastase, but that the action of this enzyme is enhanced by visible rays owing to activation of the zymogen. As was mentioned above, visible rays have only a slight retarding action on chymosin, catalase and peroxydase, but these enzymes are rapidly and permanently inactivated by ultra-violet radiation (Schmidt-Nielsen, Zeller and Jodlbauer). Signe and Sigval Schmidt-Nielsen made a detailed, kinetic study of the destruction of rennet by ultra-violet light (H., 1908, **58**, 235), whilst shortly beforehand Georges Dreyer and Olav Hanssen (C. R., 1907, **145**, 564) showed that the destruction of enzymes by radiation follows the law for unimolecular reactions.

Of Schmidt-Nielsen's experiments, which were made in the Finsen Institute with a mercury-vapour lamp, the following may be described:

A 1% solution of dry, commercial rennet powder was exposed to the radiation for a definite period and the time required for the coagulation of cow's milk subsequently measured; this time was assumed to be a direct measure of the amount of unaltered enzyme in the solution.

Temperature, ° C.	Exposure to the light, minutes.	Time of clotting, minutes.	1000k.
24·2	0	8·5	—
24·2	1·0	23·5	442
24·2	1·5	39·25	443
24·2	2·0	71·0	461
0	0	7·7	—
12·75	1·0	19·5	405
12·80	1·5	34·5	434
12·85	2·0	59·0	442
13·90	2·0	56·0	431
12·95	2·0	54·5	425

As was, indeed, to be expected, these numbers show that the destruction of rennet by light is undoubtedly a unimolecular reaction. But what deserves special attention is the extraordinarily small temperature-coefficient of this reaction. That such

processes possess low temperature-coefficients has been repeatedly observed and seems to be a general rule. It may be assumed in the above case that the temperature of the chymosin-molecules, after exposure to the light, is considerably higher than that of the surrounding solution and is not very different in the two series of experiments; the temperature of the solution had therefore little influence on the thermal condition of the chymosin.

In Schmidt-Nielsen's experiments, the reaction constant diminished with increase of the concentration. This is not surprising, since the destruction by heat of enzymes proceeds more slowly in their concentrated than in their dilute solutions. Hence, as their concentration increases, enzymes become more stable to both heat and light. Of the total effect of the radiation of the mercury lamp, 96% is due to rays with the wavelengths 220–250 μμ and only about 0·3% to the visible rays.

Very interesting experiments have been made by von Tappeiner and his co-workers on the action of sunlight on diastases and invertase in presence of fluorescent substances (sensitisers). Very small quantities of eosin, Magdala red or quinoline red are sufficient to cause sunlight—which of itself is with action—to exert a marked inhibiting effect.

In diffuse daylight the fermentative power of yeast is destroyed by fluorescent bodies. With living yeast, only certain fluorescent substances are active; but with permanent acetone-yeast and, still more, with yeast-juice, all the fluorescent bodies examined, such as eosin, methylene blue, fluorescein, dichloranthracene-disulphonic acid, etc., induce considerable diminution of the fermentative power (von Tappeiner, Biochem. Z., 1908, **8**, 47).

Also on catalase all the fluorescent substances investigated have a sensitising action, whilst with peroxydase this is the case only with eosin and Bengal red; in both these instances, the action only occurs when the ultra-violet rays are, as far as possible, lacking (Jamada and Jodlbauer, Biochem. Z., 1908, **8**, 61; Zeller and Jodlbauer, ibid., 84; Karamitsas, Dissertation, Munich, 1907).

Thus the biological action of light is of two kinds (Jodlbauer and von Tappeiner, Deut. Arch. f. klin. Med., 1906, **85**, 386): One requiring the presence of oxygen and accelerated by fluorescent substances, and the other produced

only by ultra-violet rays without any part being played by oxygen or fluorescent substances.

The results mentioned above show that light exerts actions of two kinds on enzymes:

(1) A destroying action, corresponding with denaturation by heat.

(2) An activating effect, due to conversion of the " zymogen " into the active enzyme.

2. Other Forms of Radiation

By Röntgen rays, enzymes are not weakened. This was shown by P. F. Richter and Gerhartz (Berl. klin. Wochens., 1908, **45**, 646) to be the case with chymosin, yeast, pepsin, pancreatin and papain, while Lockemann, Thies and Wichern obtained the same result with blood-catalase.

Radium rays and radium emanation, however, do appear to exert an action on enzymes, although, according to Wilcock (Journ. of Physiol., 1907, **34**, 207), tyrosinase is not affected by radium rays, while Schmidt-Nielsen found that even a very active preparation of radium has a very slight effect on chymosin; Henri and Mayer (C. R., 1904, **138**, 521) state that invertase, emulsin and trypsin are injured. Against these negative assertions are arrayed a number of other positive results.

Bergell and Bickel (Verhandl. d. Kongr. f. inn. Med., Wiesbaden, 1906) first showed that peptic digestion is favoured by the emanation. Neuberg (Verhandl. d. deutsch. path. Ges., 1904) and Wohlgemuth (ibid) observed acceleration of the autolytic processes by radium radiation, while Loewenthal and Edelstein (Biochem. Z., 1908, **14**, 484) found these processes to be facilitated by the emanation. Also Loewenthal and Wohlgemuth (Biochem. Z., 1909, **21**, 476) have recently proved that radium emanation is capable of accelerating the action of the diastatic enzyme of the blood, liver, saliva or pancreas. " This favourable action is not always observable immediately; very often retardation occurs during the first 24 hours, this being gradually neutralised and then replaced, if the experiment is sufficiently prolonged, by an acceleration. In other cases, the emanation produced only

inhibition, which was not compensated when the duration of the experiment was extended."

Acceleration of enzyme-action by the emanation has been established with pepsin and trypsin.

Of importance from a therapeutic standpoint is G u d z e n t ' s discovery that the enzyme of purine-metabolism is activated by radium emanation.

Action of m e s o t h o r i u m .

The mesothorium bromide discovered by O. H a h n emits rays of three kinds: α-, β- and γ-rays. Of these, the β- and γ-rays pass without alteration through a mica plate, if this is not too thick; they are also able to traverse a thin sheet of glass, but are then weakened to some extent. B i c k e l and M i n a m i (Berl. klin. Wochens., 1911, **48**, 1413) found that exposure of carcinoma, sarcoma and liver to the radiation of mesothorium bromide—the action of emanation or α-rays being excluded—has no influence on the autolytic enzymes. These authors regard their results as of fundamental importance. If it is a fact that the β- and γ-rays of mesothorium are identical in every respect with the β- and γ-rays of radium, it must be concluded that the activation of the autolytic enzymes observed as a result of the action of radium is s o l e l y an effect of the α-rays or emanation. The same is probably the case with other enzymes. According to M i n a m i (Berl. klin. Wochens., 1911, **48**, 1798) the β- and γ-rays of mesothorium exert a very slight influence on the digestive enzymes, amylase, pepsin and trypsin. The biological action of thorium emanation has been studied by B i c k e l (Berl. klin. Wochens., 1911, **48**, 2107); like that of radium emanation, it consists sometimes of a retardation and sometimes of an activation of enzymic action, and is more intense than that of β- and γ-rays.

In general, it may be stated that the healing action found by experience to be exerted by radium emanation depends on the activation of enzymes. The promotion of plant-growth by the emanation (F a l t a and S c h w a r z , Berl. klin. Wochens., 1911, **48**) is also to be attributed to enzyme-activation.

CHAPTER VI

CHEMICAL STATICS IN ENZYME REACTIONS

The position of equilibrium of a chemical system is determined, as is well known, by the law of mass action.

If 1 mol. of acetic acid reacts with 1 mol. of alcohol, so that 1 mol. of ester and 1 mol. of water are formed, then, according to the law of mass action·

$$\frac{[\text{ester}]}{[\text{acid}][\text{alcohol}]} = K,$$

if the concentrations of the substances in dilute aqueous solution are indicated by [ ] and K denotes the equilibrium constant.

According to van't Hoff, chemical equilibrium of the above reaction is due to the equality of the velocity v_1 of ester-formation and of the velocity v_2 of ester-decomposition, so that,

$$v_1 = k_1[\text{acid}]\,[\text{alcohol}] = v_2 = k_2[\text{ester}],$$

and therefore

$$\frac{[\text{ester}]}{[\text{acid}][\text{alcohol}]} = K = \frac{k_1}{k_2}.$$

The position of equilibrium is independent of the rapidity with which it is reached and also—as exact experiments show—independent of the presence and concentration of a catalyst, in so far as this does not combine to an appreciable extent with the components of the system.

As was mentioned in Chapter IV (p. 128), a number of investigators have arrived at the conclusion that enzymic reactions are effected by means of a compound of the enzyme and the substrate.

With the non-enzymic hydrolyses which have been as yet investigated and in which, according to the author's theory, the

increase of active molecules is due to the formation of a salt of the substrate with the catalysing acid, the concentration of the compound substrate-catalyst is so small that no great alteration occurs in the concentration of the substrate or catalyst, either during the course of the reaction or when equilibrium has been attained.

Enzymic decompositions usually differ from those effected by inorganic catalysts in that their velocity is determined not only by the absolute concentration of the catalyst (enzyme) but also, and to a far greater extent, by the concentration-r a t i o between enzyme and substrate. If the substrate is in excess, the velocity of reaction is approximately proportional to the concentration of the enzyme, whilst if excess of the latter is present, the velocity will be very nearly proportional to the concentration of the substance; in every case, the velocity of reaction appears to be proportional to the concentration of the "intermediate product."

On the other hand, quantitative study of enzyme-reactions has shown that the products of reaction are also fixed by the enzyme (H e n r i , B o d e n s t e i n , and others).

The question now to be considered is the relations in the case of enzymic reactions.

A distinction must here be made between true equilibria and end-states.

A. Equilibria

The assumption of the existence of the molecules enzyme-substrate and enzyme-reaction product which bring about the reaction presumes that the mutual action between these molecules proceeds far more rapidly than those between the free substrate molecules and their decomposition products. It is, hence, principally the concentrations of the molecules of the enzyme-substrate and enzyme-reaction product which condition the end-state.

The simple assumption may first be made that, in unit time, equal numbers of enzyme-substrate and enzyme-reaction product molecules take part in the reaction. The velocity constant k_1 of the d e c o m p o s i t i o n o f t h e s u b s t r a t e is

proportional to the concentration of the molecules of the enzyme-substrate compound; or if

$$K_1 = \frac{[\text{enzyme-substrate}]}{[\text{enzyme}][\text{substrate}]},$$

then

$$k_1 = K_1[\text{enzyme}]\,[\text{substrate}].$$

In a similar manner the velocity constant of the formation of the substrate is expressed by

$$k_2 = K_2[\text{enzyme}]\,[\text{reaction product}]^2,$$

if, as is often the case, 2 mols. of reaction product are formed from 1 mol. of substrate.

According to van't Hoff, the equilibrium is then given by the quotients

$$K = \frac{k_1}{k_2} = \frac{K_1[\text{substrate}]}{K_2[\text{reaction product}]^2}.$$

As will be at once seen, the numerical value of this "enzymic" end-state coincides with that of the natural stable equilibrium as reached with an inorganic catalyst, only if $K_1 = K_2$, hence only in the case where the combinations enzyme-substrate and enzyme-reaction product are exactly equal. No convincing cause has, however, yet been suggested for such an assumption; on the contrary, the results obtained by Henri with invertase bear the interpretation that this enzyme is combined equally by cane-sugar, glucose and fructose.

If the simple assumption, that equal numbers of the two "active" molecules react per unit of time, is abandoned and it is assumed that $n\%$ of enzyme-substrate and $m\%$ of enzyme-reaction product molecules react in equal times, then

$$K = \frac{k_1}{k_2} = \frac{K_1 n[\text{substrate}]}{K_2 m[\text{reaction product}]^2},$$

and K is identical with the constant of stable equilibrium if $K_1 n = K_2 m$.

Hence it will in general be expected that enzymes lead to an end-state different from that given by inorganic catalysts and

the first question to be decided is the position of the natural equilibrium. The natural equilibrium can be determined directly in many cases, for instance, in the system fatty acid-alcohol-ester-water. In order to accelerate the attainment of equilibrium, a strong mineral acid may be employed, since the position of equilibrium is not altered by such an ideal catalyst. In certain other cases, an indication of the position of equilibrium may be obtained from the heat-change of the reaction (v a n ' t H o f f , Sitzungsber. K. Akad. Berlin, 1909, **42**, 1065).

As was shown above, the heat-effects of enzymic processes are mostly very small. For such changes, however, the equilibrium is of a simple nature. " Optical antipodes which form no racemic compound present the ideal example, and it has been shown both theoretically and experimentally that, in the solid state, the two antipodes are in equilibrium, whilst in the vaporous, fused and dissolved conditions, they form an inactive mixture of equal amounts. The relation between the equilibrium constant K, i.e., the quotient of the concentrations of the two antipodes, and the work of transformation E may be expressed thermodynamically (v a n ' t H o f f , Svenska Vet. Akad. Handl., 1886) by

$$ln\ K = -\frac{E}{2T}.$$

Hence in this case $E=0$ and $K=1$.

What applies strictly to optical antipodes also holds approximately with reactions of small heat-effect and the equilibrium is not far removed from that corresponding with thermo-neutrality.

How far true e n z y m i c equilibrium may differ from the n a t u r a l equilibrium cannot be stated exactly. It will depend on the proportion of the components of the equilibrium which combines with the enzyme or—if it is assumed that separate enzymes accelerate the reaction in the two directions—two enzymes to form complex compounds. Since the concentration of the enzyme is usually very low, the concentrations of the molecules of enzyme-substrate and enzyme-reaction product must also be low, and the enzymic and natural equilibria will then differ but l i t t l e . In other words, if the corresponding enzyme or mixture of enzymes is added to a system in equilibrium, the

latter undergoes only slight change. This is required by thermodynamics, which also shows that in the case where the catalyst and the reacting substances do not (practically) unite, no change in the equilibrium should be produced. Otherwise the equilibrium could be altered by alternate removal and introduction of the catalyst and perpetual motion thus attained.

B. End-states and Stationary States

Starting with a system not in chemical equilibrium, the natural equilibrium is not necessarily arrived at by addition of an enzyme-preparation capable of acting on the system. An end-state differing from the equilibrium will be attained.

1. If two enzymes exist which catalyse the reaction in opposite directions. The final state then depends on the relative quantities of these two enzymes.

2. If two enzymes are present, one catalysing the formation of a compound A of the components by the non-reversible reaction

$$B+C \xrightarrow{\text{Enzyme 1}} A,$$

and the other using up this compound A according to another non-reversible reaction.

$$A \xrightarrow{\text{Enzyme 2}} D+F.$$

This case may evidently lead to widely varying stationary states, depending on the relative concentrations of the two enzymes 1 and 2.

Tammann (H., 1892, **16**, 271) gave an account of the experimental material obtained before 1892 and also of his own investigations. From the results of the latter he deduced the important and undoubtedly correct consequence, that the end-states of enzymic reactions do not coincide with the positions of stable equilibrium of the reactions.

Concerning the end-states attained under the influence of lipases, two more recent papers have been published:

Bodenstein and Dietz (Zeitschr. f. Elektrochem., 1906, **12**, 605) have compared the equilibrium formed between amyl butyrate, water, amyl alcohol and butyric acid with the end-state attained by this system under the influence of lipase. The measurements of the velocity-constants, k_1 and k_2, with which the formation and resolution of the ester proceed, have already been referred to on p. 152. The mean values obtained were:

$$k_1 = 0{\cdot}015 \qquad k_2 = 0{\cdot}0072$$

As should theoretically be the case, the quotient of these two velocity constants was found to be equal to the equilibrium constant determined directly.[1]

Hence

$$\frac{k_1}{k_2} = K.$$

The end-state determined in these two ways showed, however, considerable and regular deviations from the natural stable equilibrium. Thus, while the natural equilibrium constant had the value 1·96, the enzyme experiments gave the following results:

Initial concentration of the reacting substances.	K
0·05	0·45
0·10	0·74
0·20	1·12

The fact that these end-states were reached from both directions proved that they were not dependent on retardations of the reaction.

Unfortunately, these data cannot serve for proving the above relations quantitatively, since the system examined was heterogeneous (macro-heterogeneous).

A. E. Taylor (Journ. of Biol. Chem., 1906, **2**, 87) also worked with Ricinus-lipase in the form of a moderately finely-divided suspension. The substrate employed was triacetin, the triglyceride of acetic acid. The natural equilibrium was investigated with 0·5, 1·0 and 2·0% solutions of the triacetin;

[1] Since water and amyl alcohol were present in excess, the constant K simplifies to

$$K = \frac{[\text{amyl butyrate}]}{[\text{butyric acid}]}.$$

mixtures of equal volumes of these solutions and of normal sulphuric acid, left for several months, gave the values:

Initial concentration of the ester.	Composition of the equilibrated liquid.
0·5%	12% ester, 88% hydrolysed
1	18 " 82 "
2	22 " 78 "

For the enzymic end-state, the following numbers were obtained:

Initial concentration of the ester.	Composition at the end-state.
0·5%	14% ester, 86% hydrolysed
1	21 " 79 "
2	30 " 70 "

From these numbers, Taylor drew the conclusion that the enzyme does not displace the equilibrium; but the differences between these two series of numbers are so large and so regular that, in the author's opinion, they do not indicate identity of the natural and enzymic equilibria. Whether such a difference exists generally and how it depends on the concentrations of enzyme and substrate are questions of great interest, and experiments in this direction should give valuable results.

Scarcely any other quantitative determinations of enzymic end-states have been made which are comparable with the natural equilibria. From Croft Hill's results on the equilibrium between maltose and glucose, Pomeranz (Wien. Sitzungsber., II B, 1902, **111**, 554) has, indeed, calculated the equilibrium constants, which are in good agreement. From a qualitative point of view however, this end-state is by no means clear; maltose is re-formed either not at all or only in inappreciable amounts, being replaced by dextrins and isomaltose.

A paper communicated by van't Hoff, shortly before his death, to the Berlin Academy of Sciences (Sitzungsber. K. Akad. Berlin, 1910, **48**, 963), treats of the equilibrium of glucosides in presence of emulsin.

Measurements were made first with the natural glucoside salicin, in presence of solid salicin and solid saligenin. It was found that the formation of solid salicin from the solid products of hydrolysis is accompanied by an expansion in volume of 9·47 c.c. per grm.-molecule. The result was that the hydrolysis

of salicin proceeds to practical completion; the equilibrium was, however, not measurable. [Also V i s s e r (Zeitschr. f. physikal. Chem., 1905, **52**, 257) had previously obtained only indications of a synthesis of salicin.]

With arbutin and aesculin, the hydrolyses were also virtually complete.

On the other hand, the system glycerol-glucose-water-glycerolglucoside gives a measurable condition of equilibrium. Experiments were made with the molecular proportion 1 : 4 between glucose and glycerol and with increasing amounts of water. If the number of mols. of the latter is expressed by b and the fraction of the glucose changed by a, then

$$\frac{\text{glucoside}\times\text{water}}{\text{glucose}\times\text{glycerol}}=\frac{a(b+a)}{(1-a)(4-a)}=k.$$

Formation of glucose still occurred with $b=15$ and $a=0\cdot38$, the value for k being 2·6. With molecular proportions of glycerol and glucose, as much as 70% may undergo change.

T a m m a n n has investigated experimentally the dependence of the end-state on the quantities of the enzyme and of the reacting compounds. In the action of emulsin on arbutin and on coniferin, it was found that the amount of substance hydrolysed at the end-state increases to a maximum as the quantity of enzyme increases. This indicates that, with increasing concentration of the enzyme, the number of the active molecules, enzyme-product of reaction, increases more than that of the molecules enzyme-substrate.

When constant amounts of emulsin and different amounts of amygdalin are dissolved in 25 c.c. of water, the following amounts of amygdalin are decomposed at 40°:

Original quantity of amygdalin.	Amounts hydrolysed	
	Absolute amounts.	Percentage amounts.
0·51 grm.	0·11 grm.	20
1·02 grms.	0·15 "	15
2·04 "	0·24 "	12

Similar relations are found for the end-states of the system emulsin-arbutin. The solution contained 0·0625 grm. of emulsin and the following amounts of arbutin at 35°:

Original quantity of arbutin.	Hydrolysed after	
	48 hours.	72 hours.
0·576 grm.	52·3%	52·3%
4·000 grms.	44·0	44·0

With constant amounts of enzyme, relatively more amygdalin and arbutin are hydrolysed in dilute than in concentrated solutions; the same probably holds for the hydrolysis of coniferin by emulsin.

The question now arises: Within what limits is the end-state of an enzymic chemical system variable? That these limits must be quite wide is shown at once by the above facts. They depend not only on the concentration, but also on the previous history of the enzyme.

Of the equilibria of biological importance with which considerable variations have been observed, that between starch and sugar in the living plant deserves special mention. The synthesis, but not the hydrolysis of starch is largely influenced by even trifling variations of temperature or by narcosis. These phenomena may be explained in two ways:

(1) The existence may be assumed of two different enzymes, one responsible for the synthesis and the other for the hydrolysis; this is, of course, only conceivable on the supposition that different quantities of the two enzymes combine preferably with the starch or sugar, so that both starch and sugar participate in two equilibria:

$$[\text{enzyme } a][\text{starch}] = k_m[\text{enzyme } a - \text{starch}]$$

and

$$[\text{enzyme } b][\text{starch}] = k_n[\text{enzyme } b - \text{starch}].$$

If corresponding equilibria hold for the compounds of the two enzymes with the sugar, it is obvious that there arise two enzymic end-states, which may assume widely different values. Their relations, one to the other, depend only on the total concentration of the starch. None of the facts are contradictory to this hypothesis, for which, however, no experimental proof is forthcoming. In such a case, indeed, the enzymes are far removed from ideal catalysts. (2) Or it remains to be tried —as the author has emphasised in another place (Pflanzenchemie, II and III, p. 237)—whether the assumption of a single catalyst or a single

equilibrium constant does not meet the case. The very varying ways in which the opposed processes of building-up and breaking down react towards external influences, would then be attributed to the different constitutions of the m e d i a in which condensation and hydrolysis proceed. The catalyst and the reacting substances are then regarded as d i s t r i b u t e d between the aqueous cell-sap and the protoplasmic hydrosol. In the former, owing to the great excess of water present, the hydrolysis may proceed far; but in the protoplasts, which are rich in lipoids and proteins, such a relatively small proportion of water dissolves that, with a given attainable magnitude of the sugar-concentration, the opposite reversionary changes predominate.

CHAPTER VII

ENZYMIC SYNTHESES

THE suggestion expressed by v a n ' t H o f f in 1898, that enzymes are able to effect or accelerate chemical syntheses (Zeitschr. f. anorg. Chem., 1898, **18**, 1), has since then been confirmed by the results of numerous investigations.

It was in the above year that C r o f t H i l l (Journ. Chem. Soc., 1898, **73**, 634) observed a synthetic action in the case of yeast-maltase.

C r o f t H i l l found that when yeast-maltase is allowed to act for a month at 30° on a 40% solution of glucose, the reducing and rotatory powers of the solution are so altered as to indicate formation of maltose. Shortly afterwards, however, E m m e r l i n g (Chem. Ber., 1901, **34**, 600, 2207) showed that the effect observed by C r o f t H i l l depends on the formation, not of maltose, but of isomaltose and dextrinous products. Isomaltose is not again hydrolysed by maltase. Similar behaviour was noted by E . F i s c h e r and E . F . A r m s t r o n g (Chem. Ber., 1902, **35**, 3144) with kephir-lactase, which from galactose and glucose synthesises not lactose but isolactose, a carbohydrate not attacked by the lactase. Finally, E . F . A r m s t r o n g (Proc. Roy. Soc., *B*, 1904, **73**, 516) made a number of interesting observations which extended the discovery of E m m e r l i n g referred to above: the behaviour of emulsin is the opposite of that of maltase, as it hydrolyses isomaltose but synthesises glucose to maltose. These results led A r m s t r o n g to the generalisation that "Enzymes build up just those molecules which they are unable to break down."

This is a problem of fundamental theoretical importance. If A r m s t r o n g ' s view is correct, it must be assumed that those enzymes which give rise to a chemical equilibrium from both directions represent mixtures of a synthesising and a hydrolysing enzyme. Against the admissibility of this hypothesis

which is only weakly supported by experiment, no fundamental objection can be advanced, and we may again consider the facts favouring such a two-enzyme theory. It has been emphasised, especially by Bayliss, that the experimental foundation for this view is indeed rather weak, inasmuch as Croft Hill made use of ordinary brewers' yeast, which has been shown by Henry and Auld (Proc. Roy. Soc., *B*, 1905, **76**, 568) to contain " emulsin " and hence a β-glucosidase. This emulsin may have occasioned the formation of isomaltose. The formation of isomaltose and isolactose admits, however, of another possible interpretation, which has been given by E. F. Armstrong (Proc. Roy. Soc., *B*, 1905, **76**, 513).

It has been known since O'Sullivan and Tompson's work, and has been confirmed by Hudson, that the hydrolysis of cane-sugar yields a *d*-glucose distinguished by its high rotatory power; this sugar, to which Tanret—to whom we owe a very complete investigation of this sugar—gave the name α-glucose, passes gradually into ε-glucose. This ε-glucose appears to consist of α- and β-glucoses in equilibrium. In aqueous solution there is little α-glucose and a relatively large proportion of β-glucose. On adding to such a solution, an enzyme the synthetic action of which is to be studied, an excess of the β-modification is available and it is to be expected that the synthetic biose will contain the glucose-residue mainly in a form corresponding with β-glucose. The biose corresponding with the α-hexose should also be formed in smaller amount at the same time, but this has not been observed. Nothing, however, is yet known as to what constitutes the differences between these modifications.

This view is capable of experimental proof in various ways.

Firstly, it might be expected that the bioses, such as isomaltose, synthesised from glucose solutions, should yield β-glucose directly, but no such result appears to have been obtained; this should also be the case with those bioses and glucosides which undergo the same enzymic hydrolyses. Further, it would be expected that maltose could be synthesised from α-glucose—i.e., from a freshly-prepared solution of glucose—and a very active enzyme capable of effecting the synthesis, before the change from α- to β-modification is complete.[1]

[1] With reference to certain statements in the literature, it must be pointed out that originally, the names α-glucose and α-glucoside were not connected.

Apart from the facts mentioned above with reference to the synthesis of maltose and lactose, numerous other statements have been made relating to enzymic syntheses which are regarded as pure reversions.

Ethyl butyrate is formed from butyric acid and ethyl alcohol by the action of pancreas-lipase (Kastle and Loevenhart, Amer. Chem. Journ., 1900, **24**, 491).

Glyceryl butyrate (Hanriot, C. R., 1901, **132**, 212) and amyl butyrate (Bodenstein and Dietz, Zeitschr. f. Elektrochem., 1906, **12**, 605) are also formed from their components.

An extensive series of experiments on the formation of ester from methyl alcohol and oleic acid by pancreas-lipase has been carried out by Pottevin (Bull. Soc. Chim., 1906, **35**, 693; Ann. Inst. Pasteur, 1906, **20**, 901). These show, among other results, that the equilibrium between glycerol and oleic acid is independent of the quantity of enzyme added.

Quantity of pancreatin employed.	Percentage of ester formed.		
	1 day.	2 days.	20 days.
1	8	56	84
2	12	66	82
5	21	66	84
10	43	74	85

The following results, relating to the synthesis of a true fat, indicate the influence of the amount of water present.

40 grms. of oleic acid + 3 grms. powdered pancreas. Duration of experiment, 20 days. Temperature, 33°.

Amounts of		Percentage esterified.
Glycerol.	Water.	
130 grms.	0 grms.	3
120 "	10 "	77
110 "	20 "	64
100 "	30 "	51
64 "	66 "	20
28 "	102 "	5
8 "	122 "	0

Syntheses of true fats from various higher fatty acids (of (ocoanut oil, etc.) and glycerol are described by Welter cZeitschr. f. angew. Chem., 1911, **24**, 385).

Glyceryltriacetate is formed from its components by Ricinus-lipase (Taylor, Journ. of Biol. Chem., 1906, **2**, 87). Cf. p. 154.

Amygdalin is formed from mandelonitrile glucoside and glucose (Emmerling, Chem. Ber., 1901, **34**, 3810).

Benzaldehydecyanohydrin results from benzaldehyde and hydrocyanic acid (Rosenthaler, Biochem. Z., 1908, **14**, 238; 1909, **19**, 186).

Triacetylglucose is given by acetic acid and glucose under the influence of pancreatin (Acree and Hinkins, Amer. Chem. Journ., 1902, **28**, 370).

Glycogen is formed in pressed yeast-juice from sugar (Cremer, Chem. Ber., 1899, **32**, 2062).

A condensation, the nature of which is not clearly understood, is produced in invert-sugar solutions by the revertase of Mucor mucedo, etc. (Pantanelli, Atti Real. Accad. Lincei, 1907, [v], **16**, ii, 419; Bot. Ber., 1908, **26**a, 494). Also no definite conclusions can be drawn concerning the action of the yeast-revertase investigated by Kohl (Beitr. z. bot. Zentralbl., 1908, **23**, i, 64).

Reference must also be made here to the studies of Maquenne (Bull. Soc. Chim., 1906, [iii], 35; lecture) and of Wolff and Fernbach (C. R., 1903, **137**, 718) on the re-formation of amylose from its decomposition products; mention should also be made of the equilibrium attained under the action of malt-diastase (Moritz and Glendinning, Journ. Chem. Soc., 1892, **61**, 689). Possibly the reversion of starch in plasmolysed vegetable cells, observed by Overton (Vierteljahrsschr. d. naturf. Ges. in Zürich, 1899, **44**, 88), also represents such an enzyme action.

Kendall and Sherman (Journ. Amer. Chem. Soc., 1910, **32**, 1087) found that a state of equilibrium is also set up in the decomposition of starch by amylase (pancreas-amylase). In 1 per cent starch solution, equilibrium is attained—independently of the amounts of salt and alkali present—when the amount of maltose is about 85% of the initial weight of the starch.

The formation of hippuric acid from benzoic acid (benzyl

alcohol) and glycine by the action of kidney-extract has been observed by Abelous and Ribaut (Soc. Biol., 1900, **52**, 543), but confirmation of this result is desirable.

As regards the synthesis of protein substances, mention must first be made of the experiments on plastein-formation, which must undoubtedly be regarded as syntheses.

Danilewski established the fact that, in concentrated solutions of Witte's peptone, rennet produces characteristic protein precipitates. This phenomenon, "plastein-formation," which also occurs under the influence of pepsin preparations, was further investigated in Danilewski's laboratory and has since been examined more especially by Russian workers, for instance, Sawjalow (Centralbl. f. Physiol., 1902, **16**, 625) and Okuneff (Dissertation, St. Petersburg, 1895). Kurajeff (Hofm. Beitr., 1901, **1**, 121; 1903, **4**, 476) found papain to possess a similar coagulating property. Lawrow and Salaskin (H., 1902, **36**, 277) showed that the precipitation of concentrated Witte's peptone solutions by gastric juice occurs with albumoses of all types. Our knowledge of plastein-formation has recently (H., 1907, **51**, 1) been considerably extended by Lawrow, according to whom, not only the albumoses but also substances of the amino-acid type can be coagulated best in faintly alkaline solution. The coagulums exhibit the reactions of proteins but contain less nitrogen than these.

Plastein-formation is favoured by increasing the concentrations of the reacting solutions and occurs especially under conditions which retard the hydrolysis of proteins.

The precipitation of plasteins may possibly consist of a salting-out process.

Everything seems to indicate that Danilewski's reaction is really a synthesising action [1] of the pepsin or rennet, although true reversibility of the peptic action, i.e., re-formation of the starting material has not been proved. Rosenfeld (Hofm. Beitr., 1906, **9**, 215) has shown that the hydrolytic products of casein-plastein differ, at any rate quantitatively, from those of casein. This is proved very clearly by a more recent investigation of Henriques and Gjaldbäk (H.,

[1] Cf. also R. O. Herzog (H., 1903, **39**, 305) and A. Nürnberg (Hofm. Beitr., 1903, **4**, 543).

1911, **71**, 485), who followed the reaction by means of Sörensen's method of titration with formaldehyde.

An interesting pepsin-synthesis from the hydrolytic products of casein has been communicated by Robertson (Journ. of Biol. Chem., 1907, **3**, 95) who obtained a body which contained 3·17% P_2O_5 and which he termed "paranuclein."

Taylor succeeded in effecting an enzymic synthesis with trypsin. He hydrolysed protamine sulphate from Roccus linatus completely into its components and treated the mixture of amino-acids with trypsin obtained from the liver of the mollusc Schizothaerus Nuttalii (Journ. of Biol. Chem., 1907, **3**, 87).

The same author has recently described further experiments both with glycerine liver-extract of Schizothaerus Nuttalii and with pancreatin (Grübler's), the substrate being obtained as before from salmin sulphate. After a lapse of 4 months, the solutions, which proved to be still germ-free, were diluted with 4 parts of water, acidified with sulphuric acid and mixed with 3 parts of absolute alcohol; the control solutions showed no change, whilst the two containing enzyme gave thick white precipitates. The product was purified by means of its picrate, after which it was found to have a composition closely agreeing with that of salmin.

Indications of a synthetic action of trypsin on the hydrolytic products of casein were obtained by Bayliss (Arch. Sci. Biol. St. Petersburg, 1904, **11**, Supplement, 261), while the number of cases in which retardation of the hydrolysis by the reaction products has been observed show that a state of reversible equilibrium is assumed.

The experiments of Beitzke and Neuberg (Verh. d. deutsch. path. Ges., 1905, 160; Virch. Arch., 1906, **183**, 169) are of special interest, both in themselves and in relation to the representation of enzymic end-states given on p. 253 et seq. It was found that subcutaneous injection of emulsin (in rabbits) leads to the formation of anti-emulsin, which is able to synthesise glucose to maltose or to a disaccharide similar to maltose.

Should further experiments show that our enzyme-preparations contain in general a resolving and a synthesising constituent, the prevailing views concerning the formation and combination of anti-enzymes may require modification, the deter-

mining factor in the equilibrium between enzyme and anti-enzyme being ascribed to the chemical substrate and the products it yields.

Anti-enzymes

Like the toxines, many enzymes are able to cause production, in the living organism, of anti-bodies which retard the actions of the enzymes. Anti-enzyme action was first observed by Hildebrandt in 1893.

From their actions, anti-enzymes appear, like enzymes, to be organic catalysts. They seem to correspond with the enzymes in physical properties and also in chemical lability, although the observations in this direction are few and not very definite. They are, as far as is known, approximately as unstable towards high temperatures as the enzymes themselves. Among the most stable anti-enzymes are the anti-lipase of Ricinus which, according to Bertarelli (Centralbl. f. Bakt., 1905, I, **40**, 231), is not weakened at 70° and slowly loses its activity only at 80°. The degree of purity of the enzyme appears to be without influence on the formation of anti-body, and a co-enzyme is apparently unnecessary to this reaction.

This behaviour corresponds closely with that of the antitoxines, which are generally injured at 70°. Like enzymes, antitoxines are more stable in the dry state than in solution.

Judging from the results of physico-chemical investigations, the relations between enzymes and anti-enzymes are essentially identical with those between toxines and anti-toxines. For further information on this interesting problem, the monographs of Arrhenius and of Michaelis should be consulted. The most important facts concerning anti-enzymes are here brought together, because the problem of the reversibility of enzymic reactions is bound up with the action of anti-enzymes.

By the term anti-enzymes, in its stricter meaning, is to be understood those specifically-acting secretions produced in the organism, in presence of enzymes, by immunisation. But normal serum also contains substances which, for instance, annul tryptic action more or less completely. It is not probable, from the facts yet known, that these differ essentially from the anti-bodies

produced by immunisation, and they will therefore be discussed with the anti-enzymes. On the other hand, the thermo-stable and inorganic substances which prevent enzyme action to a greater or less extent will be termed, after Neuberg's suggestion, inhibiting agents.

Anti-steapsin was prepared by A. Schütze (Deut. med. Wochens., 1904, **30**, 308), who, after injecting steapsin, obtained in two cases rabbit-serum showing strong anti-lipolytic action. His results have more recently been confirmed by Beitzke and Neuberg (Virch. Arch., 1906, **183**, 169) and by Bertarelli.

The latter author (Centralbl. f. Bakt., 1905, I, **40**, 231) was not able to obtain the anti-enzymes corresponding with the lipases from ox-liver and ox-blood serum, but by injection of various vegetable lipases (Ricinus- and nut-lipase) he separated from dog-serum anti-lipases with specific actions. Thus the anti-ricinus-lipase influenced neither the serum-lipase, nor the liver-lipase, nor the nut-lipase.

Anti-emulsin, the first known anti-enzyme, was discovered by Hildebrandt (Virch. Arch., 1893, **131**, 12) who, like Beitzke and Neuberg (Virch. Arch., 1906, **183**, 169), immunised rabbits and precipitated the anti-body from the anti-ferment-serum with the globulin fraction. The synthetic action of this preparation is discussed later.

Immunisation with diastase may perhaps have been observed by Kussmaul (Arch. f. klin. Med., **14**), but his results are uncertain. Ascoli's work (H., 1904, **43**, 156) also led to no definite result.

Subcutaneous injection of diamalt—a commercial enzyme solution prepared from green malt—leads to the production in rabbit-serum of substances which retard the saccharifying action of diastase, while the serum originally showed an inverting action (Braun and Schütze, Med. Klin., 1907, No. 9, quoted in Biochem. Zentralbl., 1907, **6**, 389).

Saiki prepared an anti-inulinase by injection (Journ. of Biol. Chem., 1907, **3**, 395).

An anti-invertase of slight activity was obtained by Schütze and Bergell (Zeitschr. klin. Med., 1907, **61**, 366). Schütze (Zeitschr. f. Hygiene, 1904, **48**, 457) also prepared an anti-lactase by injecting kephir-lactase under the skin

of rabbits or into the breast-muscles of the dog; the anti-body appears in the serum, which in the normal state did not contain it.

Anti-pepsin. Sachs (Fortschr. d. Med., 1902, **20**, 425) immunised geese against pepsin, the serum exhibiting sufficient anti-peptic action to annul 20 times its amount of pepsin.

The anti-pepsin which was discovered by Weinland (Zeitschr. f. Biol., 1903, **44**, 45) and is regarded as a normal secretion of the gastric mucous membrane, corresponds with the normal anti-trypsin of blood-serum. It retards peptic digestion in vitro and doubtless prevents auto-digestion of the mucous membranes.

The same author has detected anti-enzymes of pepsin and trypsin in the pressed juice of Ascaris, and, according to R. O. Herzog (H., 1909, **60**, 306), the action of rennet preparations is also retarded by Ascaris juice.

Unlike these anti-enzymes, an agent which inhibits peptic action and was found by Blum in gastric juice is stable to heat.

While Bergell and Schütze tried in vain to obtain an anti-pancreatin (Zeitschr. f. Hygiene, 1905, **50**, 305), Jochmann and Kantorowicz, in a recent preliminary communication (Münch. med. Wochens., 1908, **55**, 728), refer to an anti-enzyme to pancreas-trypsin which must be identical with the anti-body of the leucocyte-enzyme. The same investigators state that blood contains at least two anti-pepsins, one of which inhibits the digestion of serum-albumin, being destroyed at 80–85°, while the other prevents the digestion of solidified hens' egg-albumin, being thermo-stable.

Attempts at immunisation against papain have as yet been unsuccessful (Bergell and Schütze, loc. cit.; von Stenitzer, Biochem. Z., 1908, **9**, 382).

Anti-tryptic Paralysors

The experiments of Hahn (Berl. klin. Wochens., 1897, **34**, 499) and those made almost simultaneously by Pugliese and Coggi (Boll. Sci. Med., 1897, **8**) first established the fact that normal serum retards tryptic digestion. Fermi had, however, previously observed that trypsin disappears soon after injection. Further work on this subject has been done by Achalme (Ann. Inst. Pasteur, 1901, **15**, 737), Camus and

Gley (Soc. Biol., 1897, **47**, 425), Charrin and Levaditi, Simnitzki and Glaessner (Hofm. Beitr., 1903, **4**, 79). This anti-tryptic action is bound up with the serum-albumin (Landsteiner, Centralbl. f. Bakt., 1900, I, **27**, 357; Cathcart, Journ. of Physiol., 1904, **31**, 497; Hedin, Journ. of Physiol., 1905, **32**, 390). With a number of diseases, such as diabetes, tuberculosis, etc., increased anti-tryptic activity of the serum is observed (Brieger and Trebing, Berl. klin. Wochens., 1908, **45**, 1041).

According to A. Döblin (Zeitschr. f. Immunitätsforsch. u. exp. Therap., 1910, **4**, 229), the anti-trypsin of serum is stable to heat, which weakens the anti-tryptic action of urine only slightly. The inhibiting body is not a lipoid but is colloidal in character.

Delezenne (Soc. Biol., 1903, **55**, 112) found that the retarding action of normal serum is not a direct action on the proteolytic enzyme, but is to be attributed to neutralisation of the corresponding kinase. He describes the following experiments:

After preliminary determination of the amount of serum just necessary to annul the digestive action of a mixture of pancreatic and intestinal juices, three tubes were each filled with equal quantities of the substance to be digested, the pancreatic-intestinal mixture and the corresponding quantity of serum. After the lapse of some hours, when it was found that no digestion had occurred in any of the tubes, to one (A) was added an excess of pancreatic juice and to another (B) an excess of gastric juice, while C served as control; digestion took place only in B. From these results Delezenne inferred that, in the digestion with the pancreatic-intestinal juice only the intestinal juice (the kinase) was neutralised, and that the serum has no action on the pancreatic enzyme. Ascoli and Bezzola (Centralbl. f. Bakt., 1903, I, **33**, 783) arrived at somewhat similar conclusions.

Against this view objections have, however, been advanced. According to Delezenne, the anti-trypsin would be an anti-kinase, and the existence of an anti-trypsinogen might also be expected. Bayliss and Starling (Journ. of Physiol., 1904, **32**, 129) were unable to detect such a body in blood-serum after subcutaneous injection. It was also shown that normal rabbit-serum possesses, in addition to its anti-tryptic properties, the ability to neutralise enterokinase, this power being enhanced

by injection of enterokinase. On the other hand, " anti-kinase " produced in serum does not increase its anti-tryptic properties.

These observations have been confirmed and extended by Zunz (Bull. Acad. Roy. Med. de Belgique, 1905, [4], 19).

Normal blood-serum contains not only anti-trypsin, but also a proteolytic enzyme, serum-protease, the action of which is retarded by the anti-trypsin. Serum-protease can be separated from anti-trypsin by salting-out, the former passing into the globulin fraction and the latter into the albumin fraction.

That the amount of anti-trypsin in serum can be increased considerably by injection of trypsin solutions, has been shown by Achalme (Ann. Inst. Pasteur, 1901, **15**, 737) and by Weinland (Zeitschr. f. Biol., 1902, **44**, 1, 45).

As regards the sensitiveness of the " anti-proteolase " to heat, Vandevelde's experiments (Biochem. Z., 1909, **18**, 142) appear to indicate that weakening takes place at 55°.

According to Fermi (Centralbl. f. Bakt., 1909, I, **50**, 225), anti-tryptic action is exhibited by various organic tissues and by certain protein substances, such as yolk of egg and milk; casein alone also has an anti-tryptic action.

The clinical significance of the tryptases and anti-tryptases, which cannot be treated in detail here, will be found discussed in a comprehensive paper by von Bergmann and Kurt Meyer (Berl. klin. Wochens., 1908, **45**, 1673).

Anti-urease. As was discovered by L. Moll (Hofm. Beitr., 1902, **2**, 344), normal (rabbit-) serum and normal albumin-free urine always exert a retarding action on urease. This action is markedly increased by injection of small doses of a urease-preparation from Micrococcus ureae Pasteuri. Moll does not regard the anti-bodies of normal and of immunised serum as identical, since the latter loses the excess of its inhibiting power and hence becomes normal in this respect if heated for an hour at 65° (but not at 56°), whilst the retarding capacity of normal serum is not altered by heating either for an hour at 65° or for six hours at 56°.

Anti-bodies of clotting enzymes. The important discovery of Morgenroth, that subcutaneous injection of rennet produces an anti-rennet in the serum and milk of the immunised animal (Centralbl. f. Bakt., 1899, **26**, 349; 1900, **27**, 721), directed attention to these anti-enzymes.

As regards anti-rennet itself, Morgenroth and more especially Madsen and Walbum, and Bashford have investigated its action quantitatively, whilst Fuld and Spiro (H., 1900, **31**, 132) have made a comprehensive study of the rennetic and anti-rennetic action of the blood. Arrhenius has pointed out the analogy existing between the behaviour of the clotting enzymes and that of certain precipitins towards the anti-bodies. It is hence unnecessary to describe the equilibrium phenomena between rennet or the fibrin-ferment and the anti-bodies.

Emphasis must be laid on the fact discovered by Hammarsten and Rödén as early as 1887 that the normal serum of various animals contains a substance which inhibits the action of rennet. This substance is, however, not identical with that produced by active immunisation (Bashford, Journ. of Pathol., 1902, **8**, 52). According to Fuld and Spiro, the "anti-rennet" contained in horse-blood serum is a pseudo-globulin which acts by fixing a portion of the calcium ions and so retarding coagulation; these authors separate the chymosin and anti-chymosin of normal blood by precipitation with ammonium sulphate.

For the relations between anti-pepsin and anti-rennet, see Jacoby (Biochem. Z., 1907, **4**, 471).

Anti-fibrin-ferment. Bordet and Gengou (Ann. Inst. Pasteur, 1901, **15**, 129) obtained this anti-body in the following manner: They injected guinea-pigs with normal rabbit-serum, by which means the guinea-pig-serum acquires the property of retarding the coagulation of rabbit-blood, i.e., the anti-fibrin-ferment is formed. This acts, as these authors showed, in a somewhat markedly specific manner on the sera of different animals. This anti-body is not affected by heating to 55°.

The composition of the anti-fibrin-ferment is not definitely known.

Anti-laccase was thought to have been obtained by Gessard (Soc. Biol., 1903, **55**, 227) in rabbit-serum by injection of laccase; the shortness of his communication renders criticism of the work impossible.

The anti-catalase of Battelli and Stern (Soc. Biol., 1905, **58**, 235, 758) behaves like ferric sulphate and should be

classed with the inhibiting agents. A true anti-enzyme of catalase does not appear to exist (De Waele and Vandevelde, Biochem. Z., 1908, **9**, 264).

Although anti-enzymes corresponding with vegetable enzymes have often been produced in the animal body (Magnus and Friedenthal), no appreciable formation of anti-enzymes in plants has yet been observed.

The most remarkable property of the anti-enzymes, in the narrow meaning of the word, is the rigid specificity of their action; this property they possess in common with the anti-toxines. Whether this is a peculiarity of the anti-bodies formed as protective agents in the organism, or whether closer investigation will show that the anti-enzymes act specifically only in the same sense as do the enzymes, cannot at present be decided.

It can, however, be asserted from the experimental material at present available, that the specificity of the enzymes does not differ fundamentally from that of other catalysts.

CHAPTER VIII

SPECIFICITY OF ENZYME ACTION

The question of the acceleration of one and the same reaction by different enzyme-preparations does not lend itself to a critical examination, owing to the impossibility of judging the physiological purity of the preparations. If therefore it is stated, for example, that emulsin hydrolyses fats, this can scarcely have any other meaning than that the preparations considered contain lipases, unless indeed it is shown that the actions on glucosides and fats always exhibit parallel courses. Meanwhile, the deciding factors in such cases are physiological in nature, whilst the chemist is concerned more nearly with the other problem, namely, that of determining what different reactions are always initiated by one and the same enzyme-preparation and enzyme.[1]

The investigations of recent years have rendered it probable that in many enzyme-preparations in which formerly the presence of only one enzyme was assumed, a number of different enzymes exist with more restricted spheres of action. It has, indeed, been mentioned that emulsin contains at least five enzymes, and that diastase is presumably composed of a number of enzymes which effect the degradation of starch in stages.

The term specificity is applied to cases where the action of an enzyme is exerted only on separate representatives of a larger class of bodies.

Some of these cases are understood in so far as the course of the chemical change can be followed. Thus, the well-known fact that only sugars with six or nine carbon atoms, and not, for instance, the pentoses, are fermentable, is much less remark-

[1] Slator (Zeitschr. f. physikal. Chem., 1903, **45**, 513) has studied the interesting case in which different, inorganic catalysts (stannic chloride, iodine chloride, etc.) cause or accelerate catalytically different reactions of the substrate (chlorine and benzene).

able now that a representation of the intermediate products of the reaction has been attained.

The specificity of the oxydases, to which attention has been repeatedly drawn, would most readily arise as a consequence of purely chemical facts.

In the cases where the specific nature of the oxydases is most pronounced, namely, with the phenolases, it may be asserted that the reactivity of the simple and substituted mono-, di- and tri-phenols is dependent on the constitution in the same way when "oxydases," as when non-enzymic manganese compounds, form the oxidising catalysts.

To choose the simplest example: of hydroquinone, pyrocatechol and resorcinol, the first is oxidised rapidly and the second considerably more slowly, whilst the last is extremely resistant to oxidation (cf. Bertrand, Bull. Soc. Chim., 1896, [iii], **15**, 791).

Another case is that of the lipases. From the results of Kastle and Loevenhart's measurements, it is known that the ester-resolving action of pancreas-extract is by no means exerted on all esters. Apart from the fact that the true fats are only very slightly hydrolysed by this extract, enormous differences are observed between the velocities of hydrolysis of such closely-allied chemical individuals as ethyl acetate and ethyl butyrate. A further list of similar differences has been given by H. E. Armstrong and Ormerod. But with catalytic decompositions, completely analogous behaviour is shown. For example, according to R. Löwenherz, the constants of hydrolysis (with hydrochloric acid as catalyst) of ethyl formate and methyl benzoate are in the ratio of 1·1 : 0·0003, and still larger differences can easily be found. In this connection, it is to be noted that in enzyme reactions very small velocities do not show, since the extended duration of the action results in the enzyme becoming inactive.

Also the results of H. Bierry and Giaja's experiments (C. R., 1908, **147**, 268) on the action of maltases and lactases of various origins appear to depend on differences of degree, and not of kind, in the activities; the resolution of lactose, lactobionic acid and lactosazone is effected by an active preparation, whilst another preparation which resolves only lactose must be generally weaker.

Fischer and Abderhalden (H., 1905, **46**, 52; 1907, **50**, 264) have collected a large mass of data concerning the power possessed by pancreatic juice of decomposing polypeptides. The behaviour of these substances towards Pawlow's pancreatic juice is shown in the following table:

Hydrolysable.	Non-hydrolysable.
*Alanylglycine [1]	Glycylalanine
*Alanylalanine	Glycylglycine
*Alanylleucine A	Alanylleucine B
*Leucylisoserine	Leucylalanine
Glycyl-*l*-tyrosine	Leucylglycine
*Alanylglycylglycine	Aminobutyrylglycine
*Leucylglycylglycine	Aminobutyrylaminobutyric acid A
* Glycylleucylalanine	Aminobutyrylaminobutyric acid B
* Alanylleucylglycine	Aminoisovalerylglycine
Dialanylcystine	Glycylphenylalanine
Dileucylcystine	Leucylproline
Tetraglycylglycine	Diglycylglycine
Triglycylglycine ester (Curtius's biuret base)	Triglycylglycine
	Dileucylglycylglycine

The hydrolysis of these substances by acids would, no doubt, likewise reveal considerable differences between the velocities. But, as Kastle and Loevenhart's experiments with esters show, the order is not always the same for hydrolysability by enzymes and by other catalysts. The lack of parallelism between the two cases may be due to enzymes and acids being combined to different extents by different esters.

The enzymes exhibit the strictest specificity towards optical antipodes.

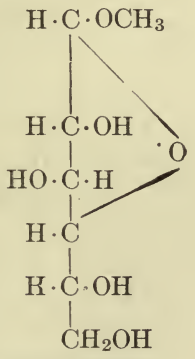
After E. Fischer had shown how new optically active products are obtained by purely chemical syntheses (Chem. Ber., 1894, **27**, 3230), the fundamental difference assumed by Pasteur between natural and artificial syntheses fell to the ground. Four years later Fischer arrived at the conclusion that the specificity of enzymes towards optical antipodes is conditioned by the stereochemical structure of the enzymes (H., 1898, **26**, 60).

[1] Peptides marked * are the racemic compounds.

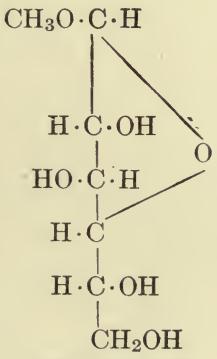
He himself, partly in conjunction with his collaborators, obtained in the hydrolysis of the methylglucosides the most striking examples of the influence of configuration on the attackability of a substrate.

Both α- and β-methyl-*d*-glucosides are acted on by enzymes, but α- and β-methyl-*l*-glucosides remain unchanged. While, however, α-methyl-*d*-glucoside is hydrolysed only by yeast-enzymes, emulsin attacks β-methyl-*d*-glucoside alone.

In general, it appears that α-glucosides are decomposed by maltase and β-glucosides by almond-emulsin.



α-methylglucoside.



β-methylglucoside.

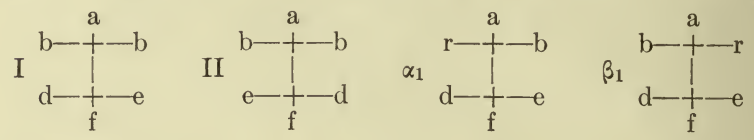
Further cases in which enzymes hydrolyse stereoisomeric compounds with very unequal velocities are given by the investigations of Fischer and of Abderhalden on polypeptides. Some of their results are as follows:

Hydrolysed.	Not hydrolysed.
d-Alanyl-*d*-alanine	*d*-Alanyl-*l*-alanine
d-Alanyl-*l*-leucine	*l*-Alanyl-*d*-alanine
l-Leucyl-*l*-leucine	*l*-Leucylglycine
l-Leucyl-*d*-glutamic acid	*l*-Leucyl-*d*-leucine
	d-Leucyl-*l*-leucine

Since the view was advanced that enzymes act as optically active catalysts, numerous cases of enzymic, asymmetric syntheses and decompositions have been observed.

Asymmetric Syntheses. If a symmetrical molecule gives rise to an asymmetric one, the dextro- and laevo-

modifications are formed in equal quantities, so that an inactive, racemic preparation is obtained. It is, however, otherwise if a molecule which is already asymmetric is employed for further syntheses. If in one of the two optical antipodes, I and II, say, in I, one of the substituents *b* is replaced by another

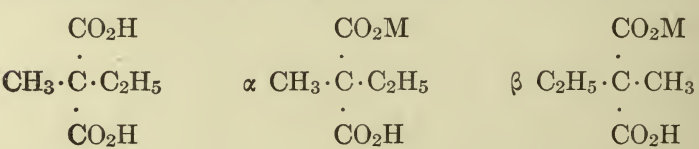


radicle *r*, two new forms, α_1 and β_1, may arise. Since in these two forms the new group *r* is at different distances from the remaining constituents of the molecule, it is evident that the molecules α_1 and β_1 will be formed with unequal velocities.

Just as two diastereomeric,[1] asymmetric products α_1 and β_1 are formed from configuration I, so also II, which is the mirror-image of I, yields two corresponding diastereomerides, α_2 and β_2, these being mirror-images to α_1 and β_1. The forms I and II thus give the two pairs of optical isomerides, $\alpha_1+\alpha_2$ and $\beta_1+\beta_2$, in different amounts.

As an example of an asymmetric synthesis analogous to those occurring in the living organism, the following case, which was investigated by Marckwald, may be taken:

Methylethylmalonic acid was converted into an acid salt:



The two forms of the acid salt, α and β, as optical antipodes' possess similar properties, except as regards the sense of their rotation. This is the case, however, only if M itself is optically inactive. But if M itself be an optically active radicle, as, for instance, if the acid brucine salt of the acid were formed, the

[1] Stereoisomeric compounds related as an object to its image in a mirror, are termed optical or enantiomorphous isomerides. On the other hand, stereoisomeric compounds which are not mirror images, one of the other, are named diastereoisomerides or diastereomerides.

two forms α and β will no longer be enantiomorphs but diastereomerides and hence will exhibit different physico-chemical behaviour. If the mixture of α- and β-forms is heated so as to remove the free carboxyl groups, the dextro- and laevo-salts must be formed in unequal amounts. The free valeric acids obtained by removal of the brucine residue, form an optically active mixture. According to Fischer's expression, from one active molecule (brucine), "another is born."

A series of interesting syntheses was also carried out by McKenzie (Journ. Chem. Soc., 1904, **85**, 1249) who, by reduction of *l*-menthyl benzoylformate with aluminium-amalgam, obtained a mixture of *l*-menthyl *d*-mandelate with a slight excess of *l*-menthyl *l*-mandelate.

Shortly afterwards, by the reduction of *l*-menthyl pyruvate, he succeeded in preparing laevo-lactic acid (Journ. Chem. Soc., 1905, **87**, 1373). With the help of Grignard's reaction, other asymmetric syntheses, such as that of laevo-atrolactinic acid from menthyl benzoylformate and magnesium menthyl iodide, were effected (Journ. Chem. Soc., 1906, **89**, 365). McKenzie and Wren prepared the optically active tartaric acids by oxidation of *d*- and *l*-bornyl and menthyl fumarates (Journ. Chem. Soc., 1907, **91**, 1215).

Of the investigations in this direction those of Dakin deserve special mention. After W. Marckwald and A. McKenzie had succeeded in showing that the velocities of esterification of two opposed optically active acids by one and the same optically active alcohol were not equal (Chem. Ber., 1899, **32**, 2130; 1901, **34**, 469), Dakin found (Journ. of Physiol., 1903, **30**, 253) that, when partially hydrolysed by lipase, inactive menthyl mandelate yields a strongly dextro-rotatory mandelic acid, while the remaining ester is correspondingly laevo-rotatory; the dextro-component of the ester is hence hydrolysed more rapidly than the laevo-component.[1] Further experiments by this investigator have led to a number of interesting conclusions (Journ. of Physiol., 1905, **32**, 199). It must be remembered that two optical antipodes, in combining with one and the same asymmetric substance, do so with unequal velocities and that, on the other hand, the products of such

[1] The hydrolysis of racemic esters has found practical application also in the preparation of optically active amino-acids (Warburg).

reactions decompose at different rates. This was the case with Dakin's asymmetric ester-hydrolysis by lipase; the latter must therefore be an optically active substance which enters into combination with the ester it hydrolyses. Experiment showed further that, in the fractional hydrolysis of a series of structurally allied racemic esters, the components which are the more rapidly attacked always possess similar configurations but not necessarily rotations of the same sign.

Asymmetric hydrolysis by means of lipase is also effected if an asymmetric carbon atom is present, not in the acid- but in the alcohol-residue of the ester. Dakin therefore drew the conclusion that combination between enzyme and ester takes place, not exclusively at the acid-group but probably with the molecule of the ester as a whole.

Finally, a number of earlier (Schulze and Bosshard, H., 1886, **10**, 134) or isolated observations, and also the more recent ones of A. McKenzie and A. Harden (Journ. Chem. Soc., 1903, **83**, 424) show that the specificity of the action of micro-organisms on optical antipodes is not complete. The enantiomorph less preferred as nutriment is also consumed by micro-organisms, although considerably more slowly and imperfectly, and even in cell-free (active) enzyme solutions, in certain cases at least, neither of the two forms seems to remain unattacked. Here also, quantitative measurements of the relative attackability of the antipodes promise valuable results.

While Dakin's experiments dealt entirely with asymmetric hydrolyses, Rosenthaler has recently described (Biochem. Z., 1908, **14**, 238) a true asymmetric synthesis, namely, the formation of *d*-benzaldehydecyanohydrin from benzaldehyde and hydrocyanic acid under the influence of emulsin. Of his experiments the following may be described:

To 5 grms. of emulsin, macerated with 20 c.c. of water, was added 0·675 grm. of hydrocyanic acid; after an hour, 20 grms. of benzaldehyde were slowly added, the liquid being kept thoroughly shaken meanwhile. The liquid was then agitated in a shaking machine for an hour, after which the nitrile was isolated and hydrolysed, and the mandelic acid extracted from the aqueous solution by means of ether. The residue from the ether, after crystallisation from benzene, showed a specific rotation of $[\alpha]_D = -153{\cdot}78°$, which is in good agreement with the value

for mandelic acid. The cyanohydrin formed was free from the laevo-form, as was shown by hydrolysis to mandelic acid.

As Rosenthaler found, in the emulsin there is a substance which conditions the asymmetry of the synthesis and another constituent which accelerates the addition of hydrocyanic acid to aldehyde or ketone. The latter of these substances proves to be a compound of magnesium, calcium or potassium. The explanation of this phenomenon is probably to be sought in Franzen's recent investigation (Chem. Ber., 1909, **42**, 3293), which showed that aldehydes and ketones, with calcium, barium, strontium or magnesium cyanide, lead to the formation of the salt of the corresponding nitrile. This reaction proceeds as follows:

$$2C_6H_5\cdot C\begin{matrix}O\\H\end{matrix} + Ca(CN)_2 = C_6H_5\cdot CH\begin{matrix}O\cdot Ca\cdot O\\CN\ \ CN\end{matrix}CH\cdot C_6H_5.$$

Of great interest is the fact that Rosenthaler (Biochem. Z., 1910, **26**, 1 and **28**, 408) has succeeded in preparing from emulsin, besides the nitrile-synthesising enzyme (σ-emulsin) which we shall term nitrilese, an enzyme which exerts solely a hydrolytic action and was named by him δ-emulsin.

By protracted heating at 40–45° the δ-emulsin is inactivated completely and the hydrolytic enzyme partially, whilst the nitrilese remains active.

Suitable treatment with acid and subsequent neutralisation with alkali also destroys the amygdalin-resolving action of emulsin, while the synthetic action (of the nitrilese) is to some extent retained.

The filtrates obtained after precipitating with copper sulphate, saturating with magnesium sulphate or half saturating with ammonium sulphate, contain no nitrilese but still hydrolyse amygdalin.

In the decomposition of amygdalin, three enzymes must hence take part (cf. p. 23): an amygdalase, a β-glucosidase and a nitrilase which resolves the mandelonitrile; the two first are hydrolysing enzymes, whilst the last has a purely decomposing action.

Rosenthaler has attempted to separate his δ-emulsin further into these three constituents in the following manner:

1. The filtrates obtained after precipitation with copper sulphate and half saturating with ammonium sulphate contained all the three enzymes.

2. The filtrate obtained after saturating with magnesium sulphate contained hydrolysing enzyme, but no nitrilase.

No other means could be discovered of separating the hydrolysing enzyme from that which decomposes the nitrile.

No satisfactory theoretical treatment of the co-existence and co-operation of a synthetic and a decomposing enzyme has, in the author's opinion, yet been advanced. Mention must, however, not be omitted of the theory developed by F a j a n s (Dissertation, Heidelberg, 1910; Zeitschr. f. physikal. Chem., 1910, **73**, 25).

An interesting case, closely related to the above, has been described by B r e d i g and F a j a n s (Chem. Ber., 1908, **41**, 752). The two optically active camphocarboxylic acids, which readily decompose into camphor and carbon dioxide when heated:

$$C_{10}H_{15}O \cdot CO_2H = C_{10}H_{16}O + CO_2,$$

do so with different velocities when they are dissolved either in pure nicotine or in a solvent containing nicotine. The following results were obtained:

Velocity of liberation of carbon dioxide in nicotine at 70°.

Per 1 grm. dextro-acid.	k_d	Per 1 grm. laevo-acid.	k_l
Dissolved in 3 c.c. nicotine .	0·00493	Dissolved in 5 c.c. nicotine .	0·00436
" 5 " .	0·00493	" 5 " .	0·00444
" 10 " .	0·00479	" 10 " .	0·00421
Mean.....	0·00488	Mean.....	0·00434

Hence in nicotine as solvent, the *d*-acid decomposes about 13% more rapidly than the *l*-acid. Salt-formation evidently takes place between the active acid and the active base, the diastereomeric bodies thus formed differing in their chemical behaviour.

In the case studied by B r e d i g , the carbon dioxide liberated is evolved, so that the nicotine previously combined with the camphocarboxylic acids becomes free after the decomposition and can form salt with fresh quantities of acid.

The difference between the experiments of Marckwald and those of Bredig consists in the employment by the latter of a weaker base. The reaction studied by Bredig hence assumes the character of a catalytic process.

Nevertheless, both the results obtained by Marckwald and those of Bredig support the view, first expressed by Fischer, that the enzymes are optically active catalysts. Their mode of action may apparently be expressed as follows:

By combination with the racemic substrate, the optically active enzymes give rise to diastereomeric substances, which decompose with different velocities and hence result in the formation of optically active material.

E. Fischer made an interesting experiment to ascertain if two oppositely active acids, *d*- and *l*-camphoric acids, hydrolyse cane-sugar with different velocities, but the result was negative. The probability of the assumption that catalysing acids, like enzymes, combine with the substrate, suggests the extension of this experiment and the making of others in which a substrate consisting of two enantiomorphs shall be decomposed by an optically active catalyst. The author has been occupied with such experiments for several years. It is evident that all facts are of value which furnish further knoweldge of the union between enzyme and substrate.

By the representation of the key fitting the lock, that is, by the hypothesis that the enzymes are optically active catalysts, the enzymes are brought into close relation with other catalysts. The development of this hypothesis is undoubtedly one of the most important aims of the chemistry of the enzymes.

CONCLUSION

What then can be given as the results of the investigation of the enzymes?

As regards the chemical nature of the enzymes, the result of our survey is but negative, inasmuch as the analyses and chemical reactions of various enzyme preparations furnish no evidence in support of the statement—often found in the literature [1]—that enzymes are protein substances; further there is nothing to indicate that all enzymes belong to a single class of substances.

[1] See, for instance, Vernon, Ergeb. der Physiol., 1910, 9, 227.

On the other hand, no fact is known which definitely disproves the protein character of any of the hydrolytic enzymes, since the results indicating the failure of enzyme solutions to give protein reactions do not give the concentrations of the solutions and have not been sufficiently controlled by means of similarly dilute solutions of undoubted proteins.

All that has yet been stated concerning the chemical constitution is mere supposition. Better than from the purely chemical investigations, we could, from the physico-chemical measurements of thermo-sensibility, i.e., from the inactivation constants, attempt to derive certain relations with the proteins, the denaturation of these by heat bearing a close resemblance to that of the enzymes. But perhaps the saponins exhibit still more marked analogies to those remarkable colloidal poisons, the physico-chemical behaviour of which is still insufficiently investigated.

The view that, for the development of their activity towards the substrate, certain enzymes require the presence of other substances—co-enzyme, acid, etc.—which are classed together as activators, has resulted in a thorough qualitative investigation of the chemistry of enzyme-action, and the consideration of these activators is of the utmost importance to the chemico-dynamic study of the enzymes.

The many deviations of the best-known enzymes, e.g., invertase, from the simple relations required by the law of mass action, had led to a formal treatment of enzymic reactions, but the results of this correspond, by no means, with the amount of labour expended. Only in the most recent times has the necessary revision of the earlier experiments been commenced; nevertheless, it cannot be regarded as premature to assert that the reactions induced by enzymes—the enzymic hydrolyses being here especially referred to—follow the laws which hold generally for catalytic reactions in solution and are deducible theoretically from the law of mass-action. Correspondence of the time-law with that for unimolecular reactions, and proportionality between concentration of the enzyme and velocity of reaction were frequently observed. Where these relations are not obeyed, the disturbing influence exerted by the products of the reaction either is known with certainty or may be assumed with a high degree of probability. In individual cases, the ultimate cause

of this disturbance is still uncertain; sometimes it must be the enzymes themselves, but in many instances the activators, which are combined, the latter case appearing to be the more common. In any case, we have seen that a simple explanation is forthcoming for S c h ü t z ' s rule; relations similar to that of S c h ü t z can also occur with inorganic catalysts, one of the best-known, apparent peculiarities of enzymic reactions thus falling to the ground. For, that the numerous " laws " such as

$$k=\frac{1}{t}\cdot\log\frac{a+x}{a-x},$$

etc., possess neither real significance nor validity may be regarded as an established and pleasing fact.

The enzymes are, therefore, catalysts. Do they, like the inorganic catalysts of the best-known reactions, for instance, hydrolysis of esters, leave the equilibrium of the reaction unchanged? Perhaps, under some circumstances and more frequently than now appears to be the case, they do so, but it can be stated with certainty that this does not always happen, and the conception of a catalyst must hence be made more comprehensive than experience of non-enzymic reactions requires.[1] But this leads to no disagreement with the principles of chemical dynamics or with the fundamental laws of thermodynamics, it being only necessary to assume that a considerable proportion of the enzyme can combine with the substrate or with the products of the reaction. The equilibrium must evidently depend on the concentration of these new molecules, enzyme-substrate or enzyme-reaction product, and any circumstance altering this concentration alters also the equilibrium or the stationary condition of the reaction.

The configuration of the substrate, the spatial arrangement of its atoms, is, as was seen in the preceding chapter, of determining importance for the occurrence of an enzymic reaction. E m i l F i s c h e r has given us the theory for these facts, the underlying assumption being that an enzyme is an optically active catalyst. This forms, with the two components of a racemic mixture, " active molecules " which are not enantiomorphous but diastereomeric products, differing in their chemical properties

[1] Cf. T a y l o r , Journ. of Biol. Chem., 1910, 8, 503.

and hence leading to the decomposition of the components with unequal velocities. Thus, what formerly appeared to characterise the catalysts of living matter presents itself, in the light of this theory, as a consequence of our views of the configuration of molecules.

But what is the nature of the combination between the enzyme and the substance succumbing to chemical attack, and how does the living organism maintain the e q u i l i b r i u m between enzyme and substrate so important to its existence? Here we meet the great riddle of the f o r m a t i o n of enzymes and anti-enzymes in the organism which opens out a region of investigation of immeasurable breadth.

APPENDIX

PRACTICAL METHODS

In what follows, a short description is given of those methods of investigating enzyme-preparations and of following enzymic decompositions which have been or might be generally applied either in medicinal practice or in industrial work.

Scientific investigation of enzymic reactions has often been effected with the acid of physico-chemical methods, but a detailed account of these would occupy too much space here, so that reference must be made to the special literature of the subject. Particular mention may be made of:

Ostwald and Luther: Manual of Physico-chemical Measurements.

W. A. Roth: Exercises in Physical Chemistry, London, 1909.

Hamburger: Osmotischer Druck und Ionenlehre in den medizinischen Wissenschaften, Wiesbaden, 1902–1904.

Also shorter references by:

H. Friedenthal, L. Michaelis, etc., in Abderhalden's Handbuch der biochemischen Arbeitsmethoden, Berlin, 1910–1912.

A. Kanitz, in Oppenheimer's Handbuch der Biochemie des Menschen und der Tiere, Band I, 1908.

As regards the methods of obtaining enzymes, these have been given in the first chapter of this book, where also the preparation of the separate enzymes in a pure state has been described.

It is only necessary here to call attention to the fact that enzymes which exert their actions within the walls of a cell are often either not at all or only very incompletely extractable, so

long as the cell-wall is living and uninjured; the dead cell-wall allows of much readier passage to the enzyme, which can thus be obtained in several ways.

1. The enzymic material is dried as rapidly, and at as low a temperature, as possible. By this treatment, the cell-walls are rendered, in some cases, more permeable and in others, more easily ruptured; these effects are enhanced if the dehydrated cells are heated at about 50–70° [cf. E. Fischer's method of preparing invertase (Chem. Ber., 1894, **27**, 2985); and Wiechowski and Wiener's method (Hofm. Beitr., 1907, **9**, 232) for preparing from the kidneys the enzyme which oxidises uric acid].

2. The finely-divided material, e.g., yeast, after being freed mechanically from water, is introduced into absolute alcohol or anhydrous acetone. Here also, the dehydration of the material by the organic solvent should be as rapid as possible.

3. The cell-walls may be destroyed by autolysis (see O'Sullivan and Tompson's method, p. 26).

4. The cells, while still living, are ruptured mechanically. The method so successfully employed by Buchner (see p. 56) is well known. Rowland (Journ. of Physiol., 1901, **27**, 53) gave a somewhat different method, in which a mixture of the cells with sand is made to assume a vigorous rotatory motion; the action resembles that of a sand-blast.

Bacteria, soft organs, etc., can be hardened by cooling in liquid air and are then readily broken up.

The analytical methods employed may now be mentioned.

Lipases. Pancreas-lipase is very suitably tested by means of an aqueous emulsion of egg-yolk, as in the work of Volhard and others. The quantity of fat hydrolysed in time t is measured and the total amount hydrolysable then calculated.

To this end, the egg-yolk emulsion containing the lipase is extracted with ether: (I) An aliquot part (50 c.c.) of the ethereal extract is titrated after addition of 50 c.c. of alcohol and then hydrolysed with 10 c.c. of normal sodium hydroxide solution, the salts of the fatty acids being decomposed after 24 hours by means of 10 c.c. of normal sulphuric acid. (II) The fatty acids

obtained by hydrolysis are estimated by titration and the percentage x of fatty acid split off by the enzyme calculated by the formula, $I : I+II = x : 100$.

In shaking the fat-emulsion with ether, more rapid separation is effected if 2–10 c.c. of alcohol are added to the ether.

In this connection see Stade, Hofm. Beitr., 1902, **3**, 291, and Engel, Hofm. Beitr., 1905, **7**, 78.

Esterases of lower esters. Ethyl butyrate is best employed as substrate. The course of the reaction is followed by direct titration or by observation of the change of the electrical conductivity.

Vegetable lipases. Ricinus seeds are skinned, freed from oil by pressing and treating the pressed cake with ether, and finely ground. The seed-juice formed is separated from the inactive constituents of the seed in a centrifuge. This juice is allowed to stand for 24 hours, during which time the enzymic emulsion, in which the acid (lactic) necessary for activation is formed, collects at the surface and can be removed. One hundred grms. of oil and 0·2 grm. of manganous sulphate are stirred up with this emulsion (5–10 grms.) and the mixture left. Here also the lipolysis can be followed by titration.

Amylases. For the estimation of the diastatic power of malt for brewery purposes, Lintner (Zeitschr. f. prakt. Chem., 1886, **34**, 386) gave the following method, which, in practised hands, gives good results.

Separate volumes of 0·1, 0·2, 0·3, ... 1·0 c.c. of malt extract [25 grms. of the ground malt+500 c.c. of water, allowed to stand at 21° (70° F.) for 3 hours and then filtered bright] are added to a series of 10 test-tubes, each containing 10 c.c. of 2% soluble starch solution, the contents of each tube being well mixed. After exactly 1 hour's rest at 21°, 5 c.c. of Fehling's solution are mixed with the liquid in each tube and the tubes then immersed in a boiling water-bath for exactly 10 minutes, after which the precipitate is allowed to settle. If the Fehling's solution in the tube containing 0·1 c.c. of malt extract is just completely reduced, the diastatic power of the malt is taken as 100; if that in the one containing 0·2 c.c. of the extract, the diastatic power is 50, and so on. A more exact result may be

obtained, if necessary, by taking 0·1, 0·15, 0·2, 0·25, etc., c.c. of malt extract for the series of tubes.

The cold-water malt extract itself contains a small amount of sugars which reduce Fehling's solution; the extent of this reduction may be determined by direct experiment, but for all ordinary purposes it is sufficiently accurate to deduct 1·5 from the value obtained for the diastatic power in the manner described above.

The following modification of the above method has been recently devised by Ling and is widely used: 3·0 (or 1·0, 2·0, or 4·0, according to the expected diastatic power) c.c. of the malt extract (prepared as already described) are added to 100 c.c. of 2% soluble starch solution in a 200-c.c. flask, the mixture being kept at 21° (70° F.) for 1 hour. At the end of this time, 20 c.c. of N/10-sodium hydroxide solution are added and the liquid made up to 200 c.c. with water. After mixing, this solution is introduced into a burette and gradually run into 5 c.c. of Fehling's solution diluted with a little water and kept boiling; this is continued until the solution just loses its blue colour or fails to give a brown coloration with a drop of ferrous thiocyanate solution on a white tile. If, say, 25 c.c. of the liquid (100 c.c. of which corresponds with 1 grm. of soluble starch and 1·5 c.c. of malt extract) are required to reduce 5 c.c. of Fehling's solution, the diastatic power of the malt will be

$$\frac{1000}{25\times 1\cdot 5}=26\cdot 7.$$

This method gives excellent results, in exact agreement with those given by Lintner's method.

With preparations of diastase, Lintner dissolves 0·2–0·5 grm. (according to the activity) in 50 c.c. of water and adds 0·1, 0·2, . . . 1·0 c.c. of this solution to a series of 10 test-tubes, each charged with 10 c.c. of 2% starch solution. The subsequent procedure is exactly similar to that employed in the case of malts.

A soluble starch which can be readily prepared with constant properties, is obtained by allowing potato starch to remain under 7·5% hydrochloric acid solution for 7 days at the ordinary temperature, then removing the acid completely by washing with cold water, and drying the starch in the air. This method

yields a product dissolving readily in hot water to a clear solution (cf. G. C. Jones, Journ. Inst. of Brewing, 1908, **14**, 13).

The determination of the velocity of hydrolysis by oxidation of the liquid with Fehling's solution (cf. Wroblewski, H., 1898, **24**, 173) is, indeed, the most reliable of the methods yet developed, although it is still capable of improvement.

A gravimetric method, which seems to give good results, has recently been proposed by Sherman, Kendall and Clark (Journ. Amer. Chem. Soc., 1910, **32**, 1073), who have also compared the older methods.

A number of other methods are based on the colorations produced by iodine in starch and dextrin solutions. Of the earlier methods, those of Detmar (H., 1882, **7**, 1) and Roberts (Proc. Roy. Soc., 1881, **32**, 145) may be referred to, whilst the following method, given by Wohlgemuth (Biochem. Z., 1908, **9**, 1), deserves special mention.

To each of a series of test-tubes containing different quantities of the enzyme solution to be tested are added 5 c.c. of 1% starch solution, each tube being placed immediately in a wire basket standing in ice-water, so as to prevent the slightest enzyme action. When all the tubes are ready, the wire basket containing them is transferred to a water-bath at 40°; by this means, the enzyme action in each tube begins at the same moment. After 30 or 60 minutes, the basket is placed again for a short time in ice-water, so that all the actions are interrupted at the same instant. The strength of the enzyme solution is then determined as follows:

All the tubes are filled with water up to within about the thickness of the finger from the top, and to each is added a drop of decinormal iodine solution, the liquid being then mixed. Different cclorations—dark blue, bluish-violet, reddish-yellow and yellow—are thus obtained. The tubes showing a yellow or reddish-yellow colour contain—disregarding further degradation of the starch to maltose or isomaltose and dextrose—only achroodextrin or erythrodextrin, the bluish-violet ones contain a mixture of erythrodextrin and starch, whilst those with a dark-blue colour contain mainly unaltered starch. As the lower limit of the activity (limes) Wohlgemuth takes the first tube in which the blue colour cannot be detected, i.e., the one showing

a violet colour. In the preceding tube, the whole of the starch is broken down to the dextrin stage at least; from this, calculation is made of the number of c.c. of 1% starch solution degraded to dextrin by 1·0 c.c. of enzyme in the time employed for the experiment.

Suppose that the tube which has become just colourless contained 0·02 c.c. of saliva; this amount was then able to transform 5 c.c. of 1% starch solution into dextrin in 30 minutes, so that 1 c.c of saliva corresponds with 250 c.c. of 1% starch solution. To indicate the diastatic power of 1 c.c. of the enzyme solution, Wohlgemuth contracts the word diastase to *D* and suggests that the temperature and duration be given for each experiment. The result of the above determination would then be stated thus: $D^{40°}_{30'}=250$. To calculate the diastatic power from an experiment in which tube No. 5 was taken as the lower limit, while tube No. 4, containing 0·0125 c.c. of saliva, was coloured red (see the scale of colour given, loc. cit.), we must proceed as follows:

In 30 minutes, 0·0125 c.c. degrades 5 c.c. 1% starch solution. In 30 minutes, 1·0000 c.c. degrades 400 c.c. 1% starch solution, so that $D^{40°}_{30'}=400$.

Other colorimetric iodine methods are given by Jungk and by Johnson (Journ. Amer. Chem. Soc., 1908, **30**, 798). A criticism of these methods will be found in Sherman, Kendall and Clark's paper (loc. cit.).

Glinski and Walther (Pawlow, Arb. d. Verdauungsdrüsen, Wiesbaden, 1898) have applied Mett's method to the estimation of diastase. Narrow glass tubes, open at both ends, are filled with starch-paste and immersed in the enzyme solution, the length of the dissolved cylinder of starch being measured after a certain lapse of time. The velocity of this reaction is evidently influenced considerably by the diffusion of the enzyme to the surface of the starch and by the rate at which the hydrolytic products are removed from this surface; movement of the liquid or of the tubes in the liquid has, therefore, considerable effect. On the other hand, this method of procedure measures the liquefaction of the starch-paste which is not a direct measure of the saccharifying action of the amylase. The method is therefore applicable only in special cases.

According to Ed. Müller (Zentralbl. f. inn. Med., 1908), the use of plates of starch-paste presents certain advantages.

Enzymes of the Disaccharides and Glucosides. In these cases, the change of the optical rotation affords a simple and accurate method of following the reaction. As was pointed out on p. 159, it is essential to destroy the mutarotation of glucose; this is best effected by the addition of soda immediately before reading the rotation.

Another method consists in determining the reducing power of the solution. This is carried out with Fehling's solution in one of a number of ways, of which that of Bertrand is one of the most accurate and convenient.

Bertrand (Bull. Soc. Chim., 1906, **35**, 1285) boils the sugar solution to be tested with Fehling's solution of definite composition for three minutes, the time being reckoned from the instant when the first bubbles form. The precipitated cuprous oxide is filtered on an asbestos filter and washed with hot water. The cuprous oxide remaining in the Erlenmeyer boiling flask and also that collected on the filter are dissolved in a solution of ferric sulphate in sulphuric acid, the following reaction occurring:

$$Cu_2O + Fe_2(SO_4)_3 + H_2SO_4 = 2CuSO_4 + H_2O + 2FeSO_4.$$

The ferrous salt is titrated with permanganate solution, standardised by means of ammonium oxalate. Bertrand has prepared tables for the most important reducing sugars, so that the amount of sugar can readily be obtained from that of the cuprous oxide formed.

The solutions employed have the following compositions:

Fehling's solution.		Iron solution.	
Copper sulphate.....	40 grms.	Ferric sulphate.....	50 grms.
Rochelle salt.....	200 "	Sulphuric acid.....	200 "
Sodium hydroxide.....	150 "	Water to.....	1 litre
Water to.....	1 litre		

Permanganate solution

5 grms. potassium permanganate per litre.

The iron solution should not reduce the permanganate. If this does occur, the permanganate solution is gradually added to the iron solution until the latter assumes a slight pink colour; it is then ready for use.

When a sugar solution is to be titrated, 20 c.c. of it are introduced into an Erlenmeyer flask of 125–150 c.c. capacity. The amount of sugar in this volume of the solution is preferably 0·010–0·090 grm. and should not exceed 0·100 grm.

To the sugar solution are added 20 c.c. of the copper sulphate solution and 20 c.c. of the alkaline tartrate solution—which are best kept separate—the liquid being then boiled for just 3 minutes after the first appearance of bubbles.

For separating the cuprous oxide, use is made of a Gooch crucible packed with asbestos and fitted as usual to a pump-flask. The precipitate is washed with hot water and as little as possible of it collected on the filter. When the washing is complete, the main quantity of cuprous oxide in the Erlenmeyer flask is dissolved in a known volume of the ferric sulphate solution; the oxide changes from bright red to blue-black and finally yields a clear, pale-green solution. This is then poured through the filter to dissolve the remaining cuprous oxide, more ferric sulphate solution being added if necessary. When all the oxide is dissolved, the Erlenmeyer flask and the filter are washed with water and the combined liquids titrated in the pump-flask with the permanganate solution. The change in colour from green to pink is extremely sharp.

The equation given above shows that 2 atoms of copper correspond with 2 mols. of ferrous sulphate and hence with 2 atoms of iron to be oxidised by the permanganate. The iron-titre of the permanganate has thus only to be multiplied by the ratio,

$$63{\cdot}6 : 55{\cdot}9 = 1{\cdot}1377,$$

in order to obtain the amount of copper, the corresponding quantity of the sugar being then given by the tables.

The permanganate solution is standardised as follows: a weighed quantity of about 0·25 grm. of ammonium oxalate is dissolved in a beaker in 50–100 c.c. of water and 1–2 c.c. of pure sulphuric acid. The liquid is heated to 60–80° and the permanganate solution run in from a burette until a pink colour is obtained.

One molecule of ammonium oxalate, $(NH_4)_2C_2O_4+H_2O$ (mol. wt., 142·1) corresponds with 2 Fe and hence with 2 Cu.

Multiplication of the weight of oxalate by $\frac{63{\cdot}6\times2}{142{\cdot}1}$, i.e., by

0·8951, gives the quantity of copper corresponding with the volume of permanganate solution required to produce the pink coloration. One litre of the permanganate solution will correspond with about 10 grms. of copper.

The tables to be used with this method are given at the end of this section (pp. 306–311).

I. Bang (Biochem. Z., 1906, **2**, 271) has described a new method for the estimation of reducing sugars which may be applied to the study of enzymic processes. It depends on the fact that, in presence of potassium thiocyanate, cuprous oxide separates as white, insoluble copper thiocyanate. The description of the method is readily accessible and will not be given in detail here.

Emulsin. In discussing the possible methods for measuring the decomposition of amygdalin, Auld points out that the estimation of the sugar liberated is affected by a number of considerations. Here also, the influence of mutarotation must be removed. He employs, therefore, in the investigation referred to on p. 173, Dunstan and Henry's titrimetric method (Proc. Roy. Soc., 1903, **72**, 287) of estimating the free hydrocyanic acid by means of standard iodine solution.

The reaction proceeds according to the equation:

$$HCN+I_2=CNI+HI.$$

Excess of sodium bicarbonate is employed to combine with the hydriodic acid formed.

Proteolytic Enzymes

On account of the great importance of measurements of digestive action to pure enzymology and also to practical medicine, the number of communications dealing with methods employed in these measurements is very large.

1. An optical method was employed by E. Schütz (compare p. 175). He removed the undigested protein from the peptic albumin solutions and estimated the quantity of peptone formed from the optical rotation of the residual liquid.

Schütz and Huppert also used this polarimetric method (see p. 179) whilst Abderhalden and Koelker

have recently studied the action of tryptic enzymes on optically active polypeptides by direct measurement of the change of rotation of the solution.

1b. Obermayer and Pick (Hofm. Beitr., 1905, **7**, 331) attempted to apply the alteration of the refractive index to the study of enzymic reactions. But this magnitude changes only in the case of tryptic digestion.

Grützner (Pflüg. Arch., 1874, **8**, 452; 1905, **106**, 463) has devised a colorimetric method. Fibrin, which has been softened by immersion in 0·1% hydrochloric acid containing carmine, is distributed as uniformly as possible into test-tubes of equal diameters, each containing 15 c.c. of 0·1% HCl, the pepsin solution to be tested being then added. The fibrin stained with carmine is dissolved and thus reddens the liquid; the intensity of the red colour indicates approximately the extent of the digestion. The digested liquid is compared with a number of tubes containing carmine solutions of definite dilutions, which are so chosen that the pepsin-content is proportional to the numbers of the colour scale. This method has been modified by Roaf (Bio-Chemical Journ., 1908, **3**, 188).

2. Measurement of the electrical conductivity was first employed for the study of peptic digestion by Sjöqvist (cf. p. 176). This method was subsequently largely used and was applied in investigations on tryptic digestion by Henri and by Bayliss (Journ. of Physiol., 1907, **36**, 221) and on the hydrolysis of dipeptides by erepsin (Euler, see p. 188); in the last experiments, a similar quantity of alkali solution was added to each solution in order to increase the variation of the conductivity.

3. Valid objections have been raised against the suggestion made by Spriggs (cf. p. 181) to determine the progress of proteolysis by measuring the viscosity of the protein solutions, and this method cannot be recommended.

4. A purely chemical method of general applicability and great accuracy was proposed several years ago by Sörensen (Biochem. Z., 1908, **7**, 45).

By this method, the content of protein or its decomposition products in a solution is determined from the number of free carboxyl-groups. The latter can be estimated by titration if

the free amino-groups of the protein are first combined. This is readily effected by addition of excess of formaldehyde, which unites with the amino-groups, giving methylene-compounds. The increase of carboxyl-groups represents the extent of proteolysis, which can hence be expressed by the number of c.c. of N/5-barium hydroxide solution employed in the titration. On the assumption that each carboxyl-group formed during proteolysis corresponds with one amino-group, the amount of proteolysis can also be stated as milligrams of nitrogen, this being obtained by multiplying the number of c.c. of the N/5-baryta by 2·8.

The titration is best carried out in presence of thymolphthalein as indicator, the solutions used being as follows:

(*a*) 0·5 grm. of thymolphthalein (Grübler's) dissolved in 1 litre of 93% alcohol.

(*b*) 50 c.c. of commercial formaldehyde solution are mixed with 25 c.c. of absolute alcohol and 5 c.c. of the thymolphthalein solution, N/5-baryta being then added until a faint green or blue colour results; this solution should be prepared fresh for each series of experiments.

As a control solution, 20 c.c. of boiled water are used. To this are added 15 c.c. of the formaldehyde solution (*b*) and about 5 c.c. of the baryta solution, the liquid being then titrated back with N/5-HCl until it assumes a faint blue opalescence. An addition is then made of two drops of baryta, which should change the colour to a distinct blue, and finally of two further drops, which should produce a vivid blue colour.

It is this last colour which is obtained in titrating the protein solution, 20 c.c. of which is mixed with 15 c.c. of the formaldehyde solution (*b*) and a slight excess of baryta solution; it is then titrated back with HCl until the colour is fainter than that of the control solution, the baryta solution being finally added in drops until the deep blue of the control is obtained.

For the description of the titration with phenolphthalein, the original paper must be consulted.

The methods of Volhard (Münch. Med. Wochens., 1903, No. 49) and Löhlein (Hofm. Beitr., 1905, **7**, 120) are based on methods given by Thomas and Weber, and by Meunier (1901). In both, casein is employed; Thomas and Weber dissolve 100 grms. of casein in 1900 c.c. of water with

the aid of 3·2 grms. of sodium hydroxide (=80 c.c. normal NaOH) or 5·04 grms. of hydrochloric acid (=138 c.c. normal HCl). The alkaline and acid solutions serve for the estimation of trypsin and pepsin respectively. After the digestion, the liquid is acidified, if necessary, with sulphuric acid and salted out with 20% sodium sulphate solution. After filtration, the precipitate is washed on the filter with hot water until the last trace of sulphuric acid is removed; the filter and precipitate are dried and weighed and the weight of undigested protein compared with that obtained in a blank experiment without pepsin or trypsin. The amount of protein dissolved gives a measure of the digestive power of the gastric juice examined.

In Meunier's method, the gastric juice (14 c.c.) is mixed with pure hydrochloric acid (0·4 c.c.) and casein (1 grm.). After the casein has settled, 2 c.c. of the clear liquid are removed and the content of free hydrochloric acid estimated. The remainder of the liquid with the undissolved casein is kept for 24 hours in a water-bath at 40°, the hydrochloric acid being again estimated in 2 c.c. of the filtrate. Since hydrochloric acid combines with protein during peptic digestion, the diminution in the amount of the free acid expresses the extent of the action.

Volhard employs a modification of the gravimetric method given by Thomas and Weber. As we have seen, the latter method is based on the observation that pure, unaltered casein, dissolved in the hydrochloric acid of the digest, is completely precipitated by sodium sulphate. Hence, if different quantities of the enzyme are allowed to act on similar amounts of casein solution for equal intervals of time at 40°, the precipitate produced by addition of sodium sulphate will be the smaller, the less the proportion of casein remaining undigested, i.e., the larger the proportion peptonised by the enzyme; the larger the residue, the smaller the quantity of enzyme. Thomas and Weber collect the precipitate on a tared, pleated filter-paper, wash with distilled water and dry and weigh. The difference in weight between the residues from experiments in which pepsin has, and has not been employed, serves as a measure of the peptic action.

Volhard avoids the inconvenience of this weighing by titration of the filtrate. He proceeds on the assumption that peptonisation of the casein solution is accompanied by increase

of the acidity of the filtrate, the peptone hydrochlorides being non-precipitable by sodium sulphate and hence passing through the filter. His experiments showed that, when equal quantities of the same acid casein solution without pepsin were used, the acidity of the filtrate was always constant and much smaller than corresponded with the true acidity of the original solution. This depends on the fact that, under similar experimental conditions, the casein precipitate always contains the same amount of hydrochloric acid, only the free acid passing into the filtrate. It is therefore justifiable to refer the excess of acidity over this constant value to the peptone hydrochlorides in the filtrate, and hence to regard the increase of acidity as a measure of the extent of digestion.

The undigested residues are in inverse proportion to the acidities of the filtrates.

As example may be given the following results of Volhard taken from Löhlein's paper (loc. cit.):

One hundred c.c. of casein solution, previously heated with 150 c.c. of water, were digested with 0·1, 0·4, or 0·9 c.c. of gastric juice (acidity 59 : 87) for one hour. Each solution was then made up to 300 c.c. in a graduated cylinder and precipitated with 100 c.c. of 20% sodium sulphate solution. Titration of 200 c.c. of the filtrate from the solution which contained no gastric juice in presence of phenolphthalein, gave the acidity as 19·15.

Two hundred c.c. from the other experiments gave

		Increase of acidity.
1. 0·1 c.c. gastric juice,	22·25 − 19·15 − acid of the juice	(0·043) = 3·06
2. 0·4 c.c. "	, 25·5 − 19·15 − " "	(0·17) = 6·18
3. 0·9 c.c. "	, 28·5 − 19·15 − " "	(0·387) = 8·96

The casein precipitates were collected on weighed filters, washed, completely dried, washed again and finally dried until constant in weight.

Weight of precipitate from original solution, $A = 4·104$ grms.

"	"	1	3·607 "
"	"	2	3·053 "
"	"	3	2·585 "

The amount digested is hence, by 1 (0·1 c.c.) $A - 1 = 0·497$ grm.
" " " 2 (0·4 c.c.) $A - 2 = 1·051$ grms.
" " " 3 (0·9 c.c.) $A - 3 = 1·519$ "

The proportionality between the degree of acidity of the filtrate and the digested amount determined by weighing, is shown by the quotients:

1. $\frac{0{\cdot}497}{3{\cdot}06}=0{\cdot}163.$ 2. $\frac{1{\cdot}051}{6{\cdot}18}=0{\cdot}170.$ 3. $\frac{1{\cdot}519}{8{\cdot}96}=0{\cdot}169.$

Of the investigations in which this method has been widely used, that of S. Küttner (H., 1907, **52**, 63) may be mentioned.

In the practical application of Volhard's method, the formulation of the results proposed by Volhard himself is to be recommended. The pepsin-unit is taken to be that quantity of enzyme which renders the filtrate from the whole of the casein used more acid by 1 c.c. of decinormal acid.

The digestion which would be produced by 1 c.c. of gastric juice in 1 hour is found from the experimental numbers by dividing the increase of acidity by the product of the time, t, and number of c.c. of juice employed, f. This number is to be multiplied by 2 or by 4, according as 200 or 100 c.c. of the filtrate are titrated. The values thus obtained for the increase of acidity follow Schütz's rule, the pepsin-unit being given by the formula,

$$x=\frac{v^2}{f\cdot t}.$$

Example: Suppose the acidity of 200 c.c. of the original solution, after precipitation and filtration, is 18·0, i.e., 36·0 per 400 c.c., and that of the juice, 20 c.c. per 100 c.c. of juice. Then, if in the digestion of 100 c.c. of casein solution made up to 300 c.c. with 3 c.c. of gastric juice for 3 hours, 200 c.c. of the filtrate obtained after adding 100 c.c. of sodium sulphate solution show an acidity of

$$32{\cdot}7(=65{\cdot}4 \text{ per } 400 \text{ c.c.}),$$

the calculation is as follows:

less 36·0, for the original solution,
and less 0·6, for the gastric juice

$$v=28{\cdot}8$$

$$\sqrt{x}=\frac{28{\cdot}8}{3\times 3}=3{\cdot}2, \quad \text{and} \quad x=10{\cdot}24 \text{ pepsin-units.}$$

Two simple methods, apparently well suited to the clinical estimation of pepsin, are due to Jacoby and Fuld.

According to Jacoby's method (Biochem. Z., 1906, **1**,

58), 0·5 grm. of ricin is dissolved in 5% sodium chloride solution and filtered. An opalescent solution is obtained which becomes turbid on addition of decinormal hydrochloric acid. Equal volumes of this solution are mixed with diminishing quantities of differently diluted gastric juice and then made up with distilled water or boiled gastric juice to a constant volume. After 3 hours in a thermostat, the liquids are examined to ascertain the smallest quantity of gastric juice able to clear the solution, i.e., to digest the protein present completely.

If, after dilution of the juice a hundredfold, 1 c.c. is just sufficient for this purpose, the number of pepsin-units in the original gastric juice is taken as 100 (normal gastric juice contains 100–200 pepsin-units).

Fuld's method (Fuld and Levison, Biochem. Z., 1907, **6**, 473; see also Zeitschr. klin. Med., 1907, **64**, 376) is as follows: A clear boiled solution (1 : 1000) of crystalline edestin in N/300-hydrochloric acid is prepared, the edestin being thus converted into the so-called edestan.

The gastric juice to be examined is now diluted in the proportion 1 : 20 and a series of dry test-tubes charged with diminishing amounts of this diluted juice by means of a 1 c.c. pipette reading to 0·01 c.c. These tubes should have a diameter of not more than about 1 c.m., so that mixing may be avoided on subsequent addition of ammonia.

The selected amount, say 2 c.c., of edestin solution is then rapidly added to each tube and after a lapse of 30 minutes ammonia solution is poured carefully into each tube, starting with the one containing most pepsin. The tubes are then observed in incident light against a black background, the one containing the smallest amount of pepsin and showing no ring being noted.

The number of c.c. of pepsin solution or gastric juice contained in this tube is divided by the product of its dilution and the number of c.c. of edestin solution digested. If, therefore, 0·25 c.c. of the 1 : 20 concentration of the gastric juice is sufficient to prevent the formation of the ring in 2 c.c. of edestin solution, the number required is $0{\cdot}25 : 20 \times 2 = 1 : 160$. The gastric juice is then termed a 1 : 160 pepsin or is said to contain 160 pepsin-units.

The methods of Jacoby and Fuld have been repeatedly tested and found to be of general utility.

Witte (Berl. klin. Wochens., 1907, **44**, 1338) suggests in Jacoby's method a slight but not unimportant modification: before use, the gastric juice is exactly neutralised; the results are thus rendered more exact.

Also Reicher (Wien. klin. Wochens., 1907, **20**, 1508) gives these methods the preference over all the older quantitative methods. He, too, found the influence of acidity to be very considerable and agreed with Witte's proposal to neutralise the gastric juice. Solms (Zeitschr. klin. Med., 1907, **64**, 159) likewise obtained favourable results with Jacoby's method.

Einhorn (Berl. klin. Wochens., 1908, **45**, 1567) suggests the simplification of Jacoby's method by the use of an apparatus of special construction. This apparatus, which is a glass vacuum-vessel, contains water at 50–60° and a stand holding graduated test-tubes charged with the digestion mixture. The time of a test may thus be shortened to 30 minutes.

Of the researches in which Fuld and Levison's method has been successfully employed, those of Wolff and von Tomaszewski (Berl. klin. Wochens., 1908), **45**, 1051) deserve special mention.

In Mett's method, hens'-egg albumin is drawn up into a glass tube 1–2 m.m. in diameter and coagulated in the tube at 95°, lengths of about 2 c.m. of the tube, cut sharply off, being then immersed in the peptic liquid. The length of the digested cylinder of the albumin is measured after 10 hours; it should not exceed about 6 m.m. The amount of pepsin is proportional to the square of this length.

According to Nierenstein and Schiff (Archiv. f. Verd. Krank., 1902, **8**, 559), the gastric juices to be compared should be brought to equal degrees of acidity.

These methods, which are open to the objections indicated on p. 292, resemble that of Fermi founded on the solution of layers of solidified gelatine.

Hattori (Arch. internat. de Pharm. et de Thérap., 1908, **18**, 255), points out that, in general, gelatine is digested much more rapidly than coagulated albumin and that two different enzymes may be in question. Such a statement is, however, hypothetical and the criticism of Fermi's method based thereon insufficiently supported.

Estimation of Trypsin and Erepsin

Most of the methods given above for the estimation of pepsin may be used, with suitable modification, to follow tryptic digestion. This is the case with the optical methods, the conductivity method (cf. Henri, and Bayliss, p. 186), etc.

The application of Volhard's method (cf. p. 297) to the estimation of trypsin is described by Löhlein (loc. cit.); the only difference is that the hydrochloric acid is added to the casein after the digestion, whilst in the investigation of pepsin it is added before digestion.

A similar method was employed by R. Goldschmidt (Deut. med. Wochens., 1909, **35**, 522).

Jacoby's ricin method also serves for the estimation of trypsin (Biochem. Z., 1908, **10**, 229). Two c.c. of a solution of 1 grm. of Merck's ricin in 100 c.c. of 1·5% sodium chloride solution are placed in each of a series of tubes together with

0, 0·1, 0·2, 0·3, 0·5, 0·7, 1·0 c.c.

of a 1% solution of Grübler's trypsin. Water is added to bring the volume to 3 c.c. in each tube, to which 0·5 c.c. of 1% soda solution is then added. The tube without trypsin remains persistently turbid whilst the others gradually clear, that with 0·1 c.c. of trypsin becoming quite bright after 6 hours in an incubator.

Chymosin. The activity of a solution of this enzyme is estimated by determining in what dilution it just coagulates a certain quantity of milk in 30 minutes at 40° (K. Glaessner, Hofm. Beitr., 1901, **1**, 1, 24; Hammarsten, H., 1896, **22**, 103).

Since the milk used for the estimation of rennet varies very considerably as regards its chymosin-content, Blum and Fuld (Berl. klin. Wochens., 1905, **42**, 107; Biochem. Z., 1907, **4**, 62) propose to replace the milk by a preparation of milk-powder, which is prepared commercially and is of constant composition. Three grms. of the milk-powder are mixed with 9 times the weight of water in the following manner: the weighed (or measured, after pressing down and smoothing in a measure) quantity of the powder is added in small portions to, and stirred

with, sufficient distilled water to form a semi-solid paste, to which the remainder of the water is then added. On stirring, almost the whole of the powder goes into solution without heating, and this solution, which can be prepared in a couple of minutes, can be used immediately if the sediment is rejected. It will, on the other hand, keep for 3 days in an ice-chest. An addition of calcium salt which was recommended by these authors in their first communication, is unnecessary.

Twenty tubes are now charged with the following amounts:

(1) of the undiluted gastric juice,
0·10, 0·15, 0·21, 0·32, 0·46, 0·68, 1·0 c.c.;

(2) of the dilution 1 : 10,
0·10, 0·15, 0·21, 0·32, 0·46, 0·68 c.c.;

(3) of the dilution 1 : 100,
0·10, 0·15, 0·21, 0·32, 0·46, 0·68 c.c.

The last tube, contained 1·5 c.c. of the boiled gastric juice, serves as a control.

The solutions are then made up to 10 c.c. with the milk solution, so that they contain the gastric juice in dilutions varying from 1 : 10 to 1 : 10,000 (a preliminary test being thus unnecessary); the tubes are then placed in a large water-bath at 17·5°.

At the end of 2 hours, a drop of 20% calcium chloride solution is added to each of the tubes, which are then transferred to a water-bath at 40° for 5 minutes. The ratio, gastric juice : milk solution in the clotted liquid containing the least amount of the juice gives directly the rennetic value of the gastric juice and also its enzyme-content in general. A more accurate estimation can afterwards be made, either immediately or, if an ice-chest is available, on the following day.

Zymase. For technical purposes and, indeed, whenever great accuracy is not desired, the fermenting power of pressed yeast-juice or permanent yeast is determined by placing in a small Erlenmeyer flask (100 c.c.) furnished with a Meissl valve, 20 c.c. of the pressed juice, 8 grms. of cane-sugar (or 2 grms. of permanent yeast, 10 grms. of water and 4 grms. of cane-sugar), and a little toluene, the loss in weight being determined after 1, 2, 3 or 4 days at 22°. The evolution of carbon dioxide amounts to about 1–2 grms. (E. and H. Buchner and M. Hahn, Die Zymasegärung, 1903, p. 80).

For more accurate estimations, the carbon dioxide is expelled from the solution by a gentle stream of air, or the evolution of gas is allowed to take place under diminished pressure, the amount of carbon dioxide liberated being then determined volumetrically.

An excellent volumetric method has been devised by Slator (Journ. Chem. Soc., 1906, **89**, 128).

Oxydase and peroxydase. The numerous reactions which have been employed for the detection and estimation of the oxydases have been referred to on p. 59. Since, as was previously mentioned, no general method for the quantitative determination of the oxydases exists, the methods given for the study of special oxydases will not be described here. Reference may be made to the brief outlines given on pp. 220–223.

Catalase. Since the determination of the catalase-content of the blood and other liquids of the body is now one of the more common tests of physiological chemistry, the most important methods may be shortly mentioned.

With aqueous solutions of purified catalases, the undecomposed hydrogen peroxide is usually determined by titration with potassium permanganate. The most suitable concentrations of the peroxide are N/20—N/50; the solutions are acidified with sulphuric acid and titrated with centinormal permanganate. In many cases this method is, as Bredig found in his researches on colloidal metals, preferable to measurement of the volume of oxygen evolved.

Where the fluid of an organ is investigated directly, volume- and pressure-methods may possess decided advantages. A volume-method was employed by L. von Liebermann (Pflüg. Arch., 1904, **104**, 176) and more recently also by Santesson, while W. Löb (Biochem. Z., 1908, **13**, 339) has described an arrangement, which apparently allows of rapid and accurate measurement of catalase-content. In the same communication, Löb describes a pressure-method, which also serves well in certain cases.

TABLES FOR THE ESTIMATION OF SUGARS BY BERTRAND'S METHOD

Glucose

Fourth crystallisation: $[\alpha]_D = \frac{4 \cdot 07^\circ \times 50 \cdot 1 \text{ c.c.}}{1 \cdot 960 \text{ grm.} \times 2 \text{ d.m.}} = +52.^\circ$

Sugar in mgrms.	Cu in mgrms.	Sugar in mgrms.	Cu in mgrms.	Sugar in mgrms.	Cu in mgrms.
10	20·4	41	79·3	71	131·4
11	22·4	42	81·1	72	133·1
12	24·3	43	82·9	73	134·7
13	26·3	44	84·7	74	136·3
14	28·3	45	86·4	75	137·9
15	30·2	46	88·2	76	139·6
16	32·2	47	90·0	77	141·2
17	34·2	48	91·8	78	142·8
18	36·2	49	93·6	79	144·5
19	38·1	50	95·4	80	146·1
20	40·1	51	97·1	81	147·7
21	42·0	52	98·9	82	149·3
22	43·9	53	100·6	83	150·9
23	45·8	54	102·3	84	152·5
24	47·7	55	104·1	85	154·0
25	49·6	56	105·8	86	155·6
26	51·5	57	107·6	87	157·2
27	53·4	58	109·3	88	158·8
28	55·3	59	111·1	89	160·4
29	57·2	60	112·8	90	162·0
30	59·1	61	114·5	91	163·6
31	60·9	62	116·2	92	165·2
32	62·8	63	117·9	93	166·7
33	64·6	64	119·6	94	168·3
34	66·5	65	121·3	95	169·9
35	68·3	66	123·0	96	171·5
36	70·1	67	124·7	97	173·1
37	72·0	68	126·4	98	174·6
38	73·8	69	128·1	99	176·2
39	75·7	70	129·8	100	177·8
40	77·5				

Invert-sugar

A 0·5% solution was prepared by hydrolysing 4·750 grms. of cane-sugar with 50 c.c. of 2% hydrochloric acid. The solution was heated for 10–15 minutes, cooled, neutralised, and diluted to a litre.

Sugar in mgrms.	Cu in mgrms.	Sugar in mgrms.	Cu in mgrms.	Sugar in mgrms.	Cu in mgrms.
10	20·6	41	79·5	71	130·8
11	22·6	42	81·2	72	132·4
12	24·6	43	83·0	73	134·0
13	26·5	44	84·8	74	135·6
14	28·5	45	86·5	75	137·2
15	30·5	46	88·3	76	138·9
16	32·5	47	90·1	77	140·5
17	34·5	48	91·9	78	142·1
18	36·4	49	93·6	79	143·7
19	38·4	50	95·4	80	145·3
20	40·4	51	97·1	81	146·9
21	42·3	52	98·8	82	148·5
22	44·2	53	100·6	83	150·0
23	46·1	54	102·3	84	151·6
24	48·0	55	104·0	85	153·2
25	49·8	56	105·7	86	154·8
26	51·7	57	107·4	87	156·4
27	53·6	58	109·2	88	157·9
28	55·5	59	110·9	89	159·5
29	57·4	60	112·6	90	161·1
30	59·3	61	114·3	91	162·6
31	61·1	62	115·9	92	164·2
32	63·0	63	117·6	93	165·7
33	64·8	64	119·2	94	167·3
34	66·7	65	120·9	95	168·8
35	68·5	66	122·6	96	170·3
36	70·3	67	124·2	97	171·9
37	72·2	68	125·9	98	173·4
38	74·0	69	127·5	99	175·0
39	75·9	70	129·2	100	176·5
40	77·7				

Galactose

Fifth crystallisation: $[\alpha]_D = \frac{16 \cdot 03^\circ \times 25 \text{ c.c.}}{2 \cdot 5 \text{ grms.} \times 2 \text{ d.m.}} = +80 \cdot 16^\circ (t = 20^\circ)$.

Sugar in mgrms.	Cu in mgrms.	Sugar in mgrms.	Cu in mgrms.	Sugar in mgrms.	Cu in mgrms.
10	19·3	41	75·6	71	126·6
11	21·2	42	77·4	72	128·3
12	23·0	43	79·1	73	130·0
13	24·9	44	80·8	74	131·5
14	26·7	45	82·5	75	133·1
15	28·6	46	84·3	76	134·8
16	30·5	47	86·0	77	136·4
17	32·3	48	87·7	78	138·0
18	34·2	49	89·5	79	139·7
19	36·0	50	91·2	80	141·3
20	37·9	51	92·9	81	142·9
21	39·7	52	94·6	82	144·6
22	41·6	53	96·3	83	146·2
23	43·4	54	98·0	84	147·8
24	45·2	55	99·7	85	149·4
25	47·0	56	101·5	86	151·1
26	48·9	57	103·2	87	152·7
27	50·7	58	104·9	88	154·3
28	52·5	59	106·6	89	156·0
29	54·4	60	108·3	90	157·6
30	56·2	61	110·0	91	159·2
31	58·0	62	111·6	92	160·8
32	59·7	63	113·3	93	162·4
33	61·5	64	115·0	94	164·0
34	63·3	65	116·6	95	165·6
35	65·0	66	118·3	96	167·2
36	66·8	67	120·0	97	168·8
37	68·6	68	121·7	98	170·4
38	70·4	69	123·3	99	172·0
39	72·1	70	125·0	100	173·6
40	73·9				

Maltose

Third crystallisation: $[\alpha]_D = \frac{26{\cdot}10^\circ \times 25 \text{ c.c.}}{2{\cdot}5 \text{ grms.} \times 2 \text{ d.m.}} = +130{\cdot}5^\circ (t = 20^\circ)$.

The above rotation refers to the hydrate, $C_{12}H_{22}O_{11}+H_2O$, but the following tables to the anhydrous sugar.

Sugar in mgrms.	Cu in mgrms.	Sugar in mgrms.	Cu in mgrms.	Sugar in mgrms.	Cu in mgrms.
10	11·2	40	44·1	70	76·5
11	12·3	41	45·2	71	77·6
12	13·4	42	46·3	72	78·6
13	14·5	43	47·4	73	79·7
14	15·6	44	48·5	74	80·8
15	16·7	45	49·5	75	81·8
16	17·8	46	50·6	76	82·9
17	18·9	47	51·7	77	84·0
18	20·0	48	52·8	78	85·1
19	21·1	49	53·9	79	86·1
20	22·2	50	55·0	80	87·2
21	23·3	51	56·1	81	88·3
22	24·4	52	57·1	82	89·4
23	25·5	53	58·2	83	90·4
24	26·6	54	59·3	84	91·5
25	27·7	55	60·3	85	92·6
26	28·9	56	61·4	86	93·7
27	30·0	57	62·5	87	94·8
28	31·1	58	63·5	88	95·8
29	32·2	59	64·6	89	96·9
30	33·3	60	65·7	90	98·0
31	34·4	61	66·8	91	99·0
32	35·5	62	67·9	92	100·1
33	36·5	63	68·9	93	101·1
34	37·6	64	70·0	94	102·2
35	38·7	65	71·1	95	103·2
36	39·8	66	72·2	96	104·2
37	40·9	67	73·3	97	105·3
38	41·9	68	74·3	98	106·3
39	43·0	69	75·4	99	107·4
				100	108·4

Lactose

Fifth crystallisation: $[\alpha]_D = \frac{13 \cdot 63 \times 50 \text{ c.c.}}{2 \cdot 5 \text{ grms.} \times 5 \text{ d.m}} = +54 \cdot 5^\circ (t = 19^\circ)$

The above rotation refers to the hydrate, $C_{12}H_{22}O_{11} + H_2O$, but the following tables to the anhydrous sugar.

Sugar in mgrms.	Cu in mgrms.	Sugar in mgrms.	Cu in mgrms.	Sugar in mgrms.	Cu in mgrms.
10	14·4	41	56·7	71	95·4
11	15·8	42	58·0	72	96·6
12	17·2	43	59·3	73	97·9
13	18·6	44	60·6	74	99·1
14	20·0	45	61·9	75	100·4
15	21·4	46	63·3	76	101·7
16	22·8	47	64·6	77	102·9
17	24·2	48	65·9	78	104·2
18	25·6	49	67·2	79	105·4
19	27·0	50	68·5	80	106·7
20	28·4	51	69·8	81	107·9
21	29·8	52	71·1	82	109·2
22	31·1	53	72·4	83	110·4
23	32·5	54	73·7	84	111·7
24	33·9	55	74·9	85	112·9
25	35·2	56	76·2	86	114·1
26	36·6	57	77·5	87	115·4
27	38·0	58	78·8	88	116·6
28	39·4	59	80·1	89	117·9
29	40·7	60	81·4	90	119·1
30	42·1	61	82·7	91	120·3
31	43·4	62	83·9	92	121·6
32	44·8	63	85·2	93	122·8
33	46·1	64	86·5	94	124·0
34	47·4	65	87·7	95	125·2
35	48·7	66	89·0	96	126·5
36	50·1	67	90·3	97	127·7
37	51·4	68	91·6	98	128·9
38	52·7	69	92·8	99	130·2
39	54·1	70	94·1	100	131·4
40	55·4				

Mannose

Third crystallisation: $[\alpha]_D = \frac{3 \cdot 48^\circ \times 25 \text{ c.c.}}{1 \cdot 25 \text{ grm.} \times 5 \text{ d.m.}} = +13 \cdot 92^\circ (t = 21^\circ)$.

Sugar in mgrms.	Cu in mgrms.	Sugar in mgrms.	Cu in mgrms.
10	20·7	60	113·3
20	40·5	70	130·2
30	59·5	80	146·9
40	78·0	90	163·3
50	95·9	100	179·4

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